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The role of plant functional traits in organising plant-pollinator interactions.

Význam funkčních znaků rostlin v uspořádání polinačních sítí.

Doctoral dissertation

Supervisor: RNDr. Robert Tropek, Ph.D.

Advisor: Mgr. Štěpán Janeček, Ph.D.

Prague, 2021

DECLARATION OF ORIGINALITY

I declare that this dissertation has not been submitted for the purpose of obtaining the same or another academic degree earlier or at another institution. My involvement in the research presented in this dissertation is expressed through the authorship of the included publications and manuscripts. All literature sources I used when writing this dissertation have been properly cited.

Prague, 17.02.2021

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 17.02.2021

Yannick Geert Klomberg M.Sc.

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STATEMENT OF CONTRIBUTION

I declare that the research found in this dissertation was conducted in collaboration with Czech and international scientists. My personal contribution to the conception, data collection, curation, analysis and manuscript writing for each of the following chapters is truthfully described in the “chapters and author’s contributions” section.

Prague, 17.02.2021

Yannick Geert Klomberg M.Sc.

As a supervisor of this dissertation and a co-author of all involved studies and manuscripts, I declare that the contribution of the PhD-student, Yannick Klomberg, to the published, submitted and finished manuscripts can be considered more than sufficient to justify the inclusion of these papers in this PhD dissertation.

Prague, 17.02.2021

RNDr. Robert Tropek, Ph.D.

CONTENTS

CHAPTERS AND AUTHOR'S CONTRIBUTIONS

This dissertation comprises the following papers and the role of authors has been described below and summarised in Table 1.

Chapter I

Klomberg Y, Tropek R, Mertens JEJ, Kobe IN, Hodeček J, Raška J, Fominka NT, Souto-Vilarós D, Janeček Š (Manuscript). Spatiotemporal shifts in the role of floral traits in shaping tropical plant-pollinator interactions.

YK was highly involved in the conceptualisation of this study, sampling of field data and their processing; he was in charge of all analyses, and their interpretations. First versions of this manuscript were written by YK under the supervision of ŠJ.

Chapter II

Mertens JEJ, Brisson L, Janeček Š, **Klomberg Y**, Maicher V, Sáfián S, Potocký P, Delabye S, Kobe IN, Pyrcz T, Tropek R (Manuscript). Elevational and seasonal patterns of butterflies and hawkmoths in plant-pollinator networks in tropical rainforests of Mount Cameroon.

YK was strongly involved in the fieldwork and processing and management of the data. Additionally, YK was in charge of measuring floral traits, managing the trait datasets and contributed to writing of the manuscript.

Chapter III

Chmel K, Ewoma FL, Uceda Gomez G, **Klomberg Y**, Mertens JEJ, Tropek R, Janeček Š (Manuscript). Bird pollination syndrome works as the plant's adaptation to ornithophily, while nectarivorous birds do not seem to care.

YK was strongly involved in the fieldwork and processing of the "insect data". YK oversaw measuring floral traits and maintaining the trait database. Furthermore, YK contributed to writing of the manuscript.

Chapter IV

Bartoš M, Janeček Š, Janečková P, Padyšáková E, Tropek R, Götzenberger L, **Klomberg Y**, Jersáková J (2020) Self-compatibility and autonomous selfing of plants in meadow communities. *Plant Biology* 22(1): 120-128. (IF_{2019}: 2.156).

YK participated in collection of plants for the greenhouse experiment and was deeply involved in collecting floral trait data in the field. Additionally, YK was involved in the writing of the manuscript.

Chapter V

Bartoš M, Janeček Š, Janečková P, Padyšáková E, Tropek R, Götzenberger L, **Klomberg Y**, Jersáková J (2020) Are reproductive traits related to pollen limitation in plants? A case study from a Central European meadow. *Plants* 9(5): 640. (IF_{2019}: 2.632).

YK participated in the fieldwork, was deeply involved in collecting floral trait data and contributed to writing of the manuscript.

Chapter VI

Klomberg Y, Dywou Kouede R, Bartoš M, Mertens JEJ, Tropek R, Fokam EB, Janeček Š (2019). The role of ultraviolet reflectance and pattern in the pollination system of *Hypoxis camerooniana* (Hypoxidaceae). *AoB PLANTS* 11(5): plz057. (IF_{2019}: 2.238).

YK helped design the experiment and was involved in the data collection and processing. He also participated in performing of additional analyses requested during the review process and their interpretations, and led writing of the manuscript, under supervision of ŠJ.

Table 1: List of chapters in this doctoral dissertation with summary of contribution of Y. Klomberg to each chapter.

	Contribution						
Chapter	Design	Fieldwork	Data processing	Data analyses	Figure/table preparation	Manuscript preparation	Manuscript editing/ review
I	✓	✓	✓	✓	✓	✓	✓
II		✓	✓			✓	✓
III		✓	✓			✓	✓
IV		✓	✓				✓
V		✓	✓				✓
VI	✓	✓	✓	✓	✓	✓	✓

PREFACE & ACKNOWLEDGEMENTS

The time in Prague has passed fast. I came into the field of pollination ecology as a naïve tropical forest ecologist, which can still be reflected in the original title of this doctoral dissertation. I should have just listened to my favourite movie: “There's far too much to take in here. More to find than can ever be found” – The Lion King (1994). I have learned a lot over these past years and the experiences gained from not only Czech Republic, but also Cameroon, have been without a doubt something I will carry closely with me during my future. Besides meeting new people and finding lasting friendships, love and inspiration, this PhD study has given me so much more! I am grateful for all the people who have played a role in making this happen. As you can see this dissertation involved several intensive fieldwork expeditions and without the collaborations of everyone involved it would not have been possible to have reached this far. This is also the reason why you find me writing “we” more often than “I” when discussing the chapters.

My biggest thanks go to my supervisors Robert Tropek and Štěpán Janeček who made it possible for me to study pollination in the Czech Republic and in Africa and supported me along the way. Their invaluable advice and help have made my PhD studies a success. Cultural differences did cause some minor instabilities, but they have also helped me in strengthening myself as a scientist. Rob was also kind to translate the abstract in Czech.

I would like to thank everyone from our Insect Community Ecology Group, and its close collaborators (including former members), for their help and unforgettable experiences together. Two persons within this group deserve more praise. Firstly, Jan Mertens, my brother-in-arms, without whom I would not have survived my PhD. Thanks for the many hours of work together on the processing of data, database management and the time spent in the field. Secondly, Sailee Sakhalkar for her great illustrations which brighten up this dissertation!

Several people from abroad deserve a mention as well. In Cameroon, Francis “Escobar” Luma, Jacques E. Chi, Collins Njie, Francis M. Teke, John Ngoto, Eric B. Fokam, Mount Cameroon National Park staff, my brother His Royal Highness Prince Dr. Nestoral T. Fominka, my sister Mercy Murkwe, and lastly the great, but small, Ishmeal N. Kobe! I am grateful for the hours we spend together in the field and without their help we would not have been able to perform our research on Mount Cameroon. I would like to thank all the people I met at conferences (especially SCAPE) for giving me insights in their worlds and showing me, again and again, why I got into science in the first place. Additionally, I would like to thank some collaborators. Firstly, Jan Wieringa, for all your help in plant identification and during my stays in Naturalis. Secondly, Paolo Biella and Agnes Dellinger, for answering my questions and your kind help. The fellow (PhD) heroes and students also deserve a mention here. Thanks for all the board game nights, dinners, and other social events! Especially Sofia and Zuzka for opening your homes (in Italy and CZ) to me. Jordan, Abel and Sailee, thanks for your comments on earlier versions of this dissertation.

Of course, I cannot stop without mentioning my family and some people I call family. My parents and my brother Roel for their eternal support, encouragement and always being there to pick me up when I needed it. Additionally, Noor, Willem, Koen, Joep, Anja, George and Margot plus the countless other friends for warmly welcoming me home and listening to my ramblings during the short visits in the Netherlands.

Finally, I am most thankful to someone I am so happy to call my fiancé, Hana, without whom I would not have reached this joyous moment. In the many days during my sickness(es) and setbacks, she was there to cheer me up. Hani, your patience, support, love and understanding are what have brought me here. I cannot thank you enough and cannot wait for the rest of our, hopefully less stressful, life together as our little family! Therefore, I dedicate this dissertation to you and our baby boy Tomáš.

ABSTRACT

Floral traits have been a key field of research in pollination ecology. The vast extent of traits studied have shown to be of influence in attracting (or deterring) visitors. Because flowers are complex organs with complicated relationships among many traits, studies of trait combinations (so-called pollination syndromes) have often been applied to help understand and predict interactions between flowers and their visitors. However, the role of individual traits should not be overlooked, especially because their roles can differ among pollinator functional groups or vary under particular conditions.

In this dissertation I give insights on the plant-pollinator interactions, the role of selected pollination related traits in shaping these interactions, and how such traits influence the mating systems and pollen limitation. We studied these interactions on Mount Cameroon (Cameroon) and in the Iron Mountains (Železné Hory; Czechia) Protected Landscape Area, where we conducted intensive observational studies, exclusion, and hand-pollination experiments.

Our results highlighted the importance of individual traits in shaping plant-pollinator interactions and plant mating systems. Nevertheless, the role and predictive ability of floral traits differed spatiotemporally and among pollinator functional groups. Interestingly, we found a higher importance of floral traits in shaping interactions towards harsher environments. Additionally, we showed that even though the pollination syndrome hypothesis is valid for bird-visited plants, we found that birds themselves selected flowers primarily based on nectar availability.

Based on my dissertation results, I conclude that prior to questioning the validity of the pollination syndrome hypothesis, we should improve our trait-based understanding of these complex plant-pollinator interactions, including spatiotemporal and taxon-specific variations.

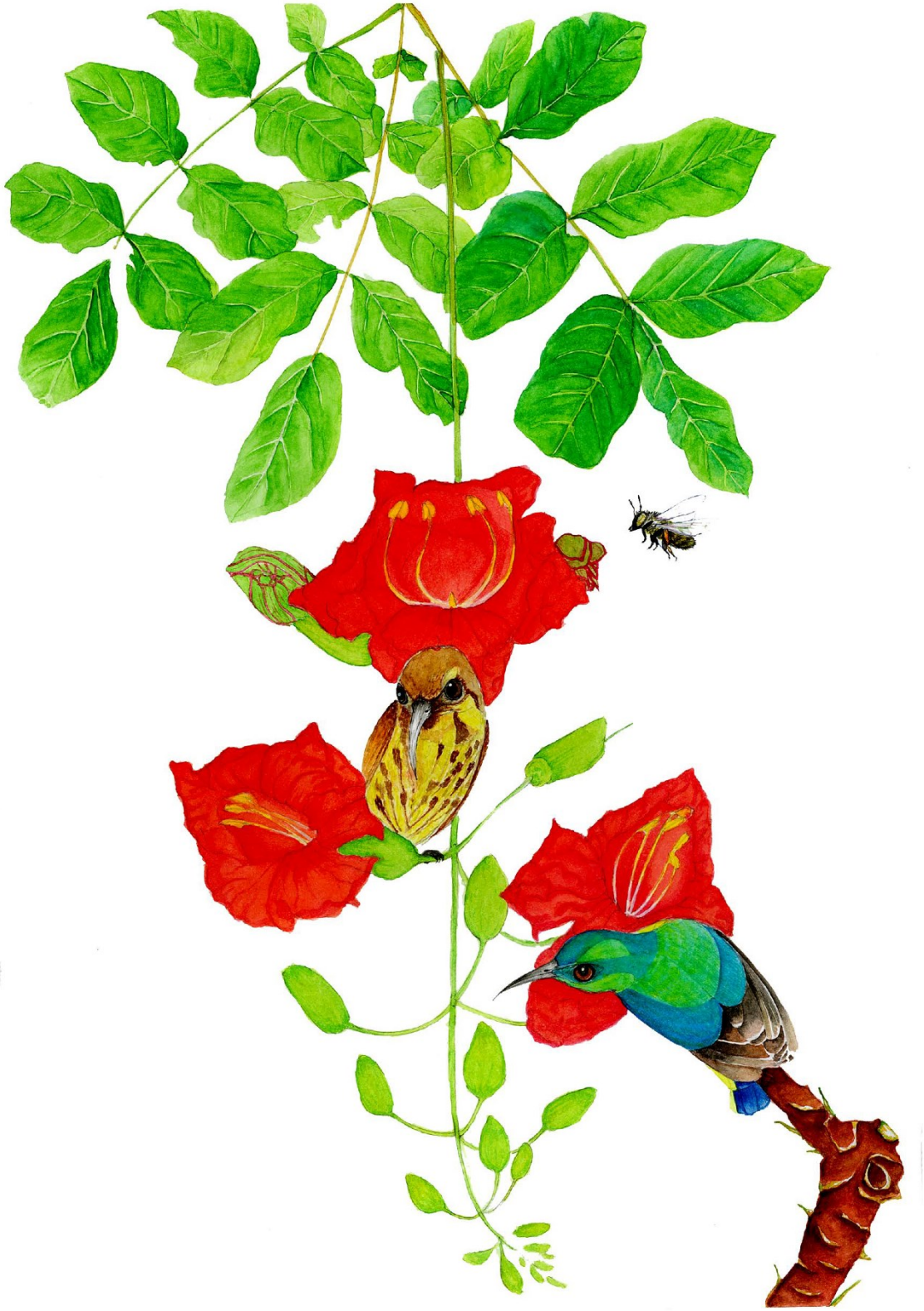
ABSTRAKT

Studium květních znaků je důležitou součástí polinační ekologie, řada z nich totiž významně přispívá k přilákání (či odlákání) návštěvníků květů. Protože květy jsou komplexními orgány s mnoha znaky propojenými složitými vztahy, interakce mezi rostlinami a jejich návštěvníky jsou často studovány pomocí kombinací květních znaků (tzv. polinační syndromy). Role jednotlivých znaků by však neměla být přehlížena, zvláště když jejich význam může být odlišný u různých funkčních skupin opylovačů, případně za různých podmínek prostředí.

V mé dizertaci se zaměřuji na interakce mezi rostlinami a opylovači, na roli vybraných květních znaků v utváření těchto interakcí a na vliv těchto znaků na reprodukční systém a pylovou limitaci rostlin. Interakce jsme pomocí rozsáhlých pozorovacích studií i experimentů s vyloučením opylovačů a s ručním opylením studovali na Kamerunské hoře (Mt. Cameroon) v Kamerunu a v Chráněné krajinné oblasti Železné hory v České republice.

Naše výsledky vyzdvihují význam jednotlivých květních znaků pro utváření polinačních interakcí i pro reprodukční systémy rostlin. Význam jednotlivých znaků se však lišil pro různé skupiny opylovačů a za různých podmínek prostředí, květní znaky byly ve studovaných interakcích nejvýznamnější zejména za nepříznivých podmínek. Také jsme ukázali, že přestože rostliny s ‚ptačím' polinačním syndromem jsou navštěvované zejména ptáky, ti si vybírají i jakékoliv jiné květy s dostupným nektarem.

Z výsledků mé dizertační práce vyplývá, že před ověřováním platnosti polinačních syndromů bychom měli lépe porozumět komplexním vztahům mezi rostlinami a jejich opylovači, zejména pak jejich proměnlivosti v čase a prostoru a specifikům různých skupin opylovačů.



INTRODUCTION

Even though my title strictly mentions floral traits, we have to take a step back first and see how these floral traits and their variability actually came to existence. Therefore, this dissertation also includes a glimpse in the history of pollination research, descriptions of plant-pollinator interactions, their specialisation, and how the plant-pollinator interactions and their coevolution have influenced the diversity in floral traits.

Pollination research: past, present and future

We need to distinguish what pollination ecology actually encompasses prior to seeing how the field has developed evolved. Pollination is the transfer of pollen from anthers to a stigma, which can be achieved abiotically (by wind or water) or biotically through a large diversity of insects, birds and mammals (Faegri & van der Pijl, 1979; Dafni, Kevan, & Husband, 2005; Willmer, 2011). It was initially thought that biotic pollination appeared with the first angiosperms (flowering plants) as early as the mid-Jurassic period (Gang *et al.*, 2016), as a replacement of the more primitive wind pollinated gymnosperms. Instead, many insect-pollinated gymnosperms had existed already before the evolutionary origin of angiosperms, for which insect pollination seems most likely the ancestral state (Ollerton, 2017, 2021). The likes of Darwin, Sprengel and Kölreuter are seen as the "founding fathers" of modern pollination ecology (Waser, 2006). However, we can go back even further to find detailed works on pollination, seed dispersal and trophic interactions by Greek (Aristotle, Theophrastus and Hippocrates) and Arab scholars (Al-Jāḥiẓ; Ings & Hawes, 2018). The role of scientific greats like Linnaeus, von Humboldt, Darwin, Wallace, Haeckel (and others), in attaining of knowledge on the interactions between plants and visitors was reviewed by Ings and Hawes (2018). Initially, the focus was on describing food chains. Humboldt's concept of "Naturgemälde" ("The artwork of nature") described the web of life, emphasising both the

relationships between species and between living organisms and their natural environment (Wulf, 2015). Darwin, largely influenced by Humboldt, built on this concept and continued working on both inter- and intra-specific competition. The field has developed since then, with the introduction of works on food cycles and trophic links (Camerano, 1880; Lindeman, 1942; Margalef, 1991), ecological niche partitioning (Elton, 1927; MacArthur, 1968), keystone species (Paine, 1966), and pollination syndromes (Delpino, 1874; Vogel, 1954; Faegri & van der Pijl, 1979). Ings and Hawes (2018) and Knight *et al.,* (2018) have extensively reviewed the advances in these fields.

Before late 20th century, most studies regarding plant-pollinator interactions were focused on partial networks, including only individual pollination systems or small groups of interacting species. This was partly due to the fact that pollination ecologists viewed plant-pollinator interactions as highly coevolved and specialised (Schluter, 2000; Knight *et al.*, 2018). A good example of this was the highly specific interaction of the long-tongued insects with flowers with long floral tubes (e.g. *Xantophan morganii*; Darwin 1877). This also led to the formation of pollination syndromes, which allows us to hypothesise a probable pollinator of a certain plant species. This is done based on a defined set of convergently evolved floral traits, such as colour, shape, and corolla tube length/width (See section Pollination syndrome hypothesis for more information). This view of highly specialised plant-pollinator interactions shifted with research examining plant-pollinator interactions as a complex network (Memmott, 1999; Vázquez & Simberloff, 2002; Olesen & Jordano, 2002; Bascompte *et al.*, 2003; Vázquez & Aizen, 2003; see next section as well).

Obviously, the study of general trends in these interactions and their characteristics at larger spatial and temporal scales will become more apparent in the near future. Although several influential scientists already published works on this subject, still work is needed to allow for such research. Currently, full network studies are rare, and many of larger scale

studies use partial networks (e.g. plant-hummingbird networks) for the goal of their research, which was so far used for trying to answer the question if networks are more specialised in the tropics compared to temperate regions (Ollerton *et al.*, 2009; Schleuning *et al.*, 2012; Trøjelsgaard & Olesen, 2013; Vizentin-Bugoni *et al.*, 2018).

However, this broader scale of research is not the only shift of focus within pollination ecology. With the increasing anthropologically-induced biotic and abiotic changes, the focus will be more on what will happen with the pollinators and their hosts, as well as how we can manage the systems to promote these important ecological keystone groups (Knight *et al.*, 2018). Additionally, more techniques have become available to analyse specific floral traits better (e.g. Spectrophotometry, Nectar carbohydrate analyses, Gas Chromatography-mass spectrometry, advanced morphometry, pollen dyeing). Therefore, I hope to see more research related to the role of individual floral traits in shaping interactions, since I believe a better understanding of the role of individual traits will greatly help our understanding of plant-pollinator interactions and pollination syndromes.

Pollination interactions and their specialisation

Pollination by animals constitutes a dominant, mostly mutualistic, ecological interaction of terrestrial habitats. Not all interactions between animals and plants are mutualistic. Plants gain pollination resources from one or multiple species of visiting animals, whereas animals gain food resources or other benefits (Waser, 2006). Both flowers and flower visitors can cheat this interaction by e.g. floral mimicry or nectar robbing/thieving. Research has shown floral diversification onto different pollinators to improve pollination effectivity (Stebbins, 1970; Waser *et al.*, 1996; Waser, 2006). Inadequate pollination of a plant can trigger selection on the plant mating system, i.e. potential shifts toward selfing, and floral traits through adaptations to improve pollen transfer (Ashman *et al.*, 2004; Knight *et al.*, 2005). Initially it

was suggested that plants through time eventually would adapt and become specialists towards a certain group of pollinators. However, it seems rather to be a paradox, where flowers could appear to be phenotypically specialised (adapted to a certain pollinator or few species of pollinators), but ecologically generalised (i.e. visited by numerous functional groups of visitors; Ollerton, 1996; Willmer, 2011). Additionally, plants are often seen to "bet-hedge". They try and attract the best pollinators (i.e. more specialised) during key moments (shortly after anthesis/dehiscence) and have enough resources available for more generalised visitors at later stages to spread the chances of successful pollen transfer (Willmer, 2011). Highly specialised plant species do occur, however often they have fallback mechanisms, such as clonality and selfing (Fenster & Martén-Rodríguez, 2007; Willmer, 2011).

Plant-pollinator specialisation (i.e. the use of a single or few plant species by a potential pollinator or a single or few pollinator species by a plant) can be defined at different levels (ecological, evolutionary and phenotypic specialisation) and has caused confusion in current literature (Armbruster, 2017). The degree of specialisation and generalisation are a fundamental aspect of any ecological interaction and the terms suggest a dichotomy, however, as mentioned before it is not that simple. ln fact, specialisation and generalisation represent two ends of a continuum in niche breadth or resource use (Waser *et al.*, 1996; Waser, 2006).

Robert MacArthur hypothesised that niche-breadth decreased towards the equator, due to an increase in interactions and less fluctuations in the environments. With niche space being partitioned due to species coexistence facilitated by specialisation (MacArthur, 1955, 1968, 1972; Vázquez & Stevens, 2004; Rasmann, Alvarez, & Pellissier, 2018). He argued that a high species richness required one or more condition to be true (being the spectrum of resources, the overlap in utilisation among species and the dimensionality of the environment being greater in the tropics, or that the niche breadths were lower in the tropics). However, Vazquéz and Stevens (2004) tested these assumptions and only found limited proof, whereas Schleuning

et al., (2012) argued that an increase in plant species diversity reduces the density of single plant species and consequently, visitors access a greater diversity of resources to reduce foraging time (i.e. more generalised systems in tropical regions). Furthermore, Pauw and Stanway (2015) showed that the latitudinal specialisation trend should probably only be attributed to the northern hemisphere. They counter the mediation of specialisation by the latitudinal gradient of plant diversity by highlighting the large plant diversity occurring together with high specialisation in South Africa (Pauw & Stanway, 2015).

Oppositely, Dalsgaard *et al.,* (2011) did show a strong link between ecological specialisation and species-rich networks, with a higher specialisation in tropical plant-hummingbird networks compared to others. In general, pollination networks possess a low connectance (i.e. a low proportion of realised interactions to all possible interactions), which is especially true in the tropics due to the high species richness and network size (Jordano, 1987; Vizentin-Bugoni *et al.*, 2018) Additionally, numerous larger scale studies have shown a similar trend with ecological specialisation being more common in the species-rich tropics (Olesen & Jordano, 2002; Armbruster, 2006; Schemske *et al.*, 2009; Trøjelsgaard & Olesen, 2013).

A circumstance when MacArthur's latitude-niche breadth hypothesis was mentioned to received support by Vázquez and Stevens's (2004) work was when species interactions were organised through asymmetric specialisation which led to a nested structure. Asymmetric specialisation is defined as: "specialised plants interacting mainly with generalised flower visitors and specialised flower visitors mainly with generalised plants" (Vázquez & Simberloff, 2002; Bascompte *et al.*, 2003; Stang, Klinkhamer, & Van Der Meijden, 2007). Asymmetric specialisation seems to be partially driven by limiting traits, such as nectar-holder depth and width (Stang, Klinkhamer, & Van Der Meijden, 2006; Stang *et al.*, 2007, 2009; Junker *et al.*, 2013). Furthermore, they show that besides size thresholds and species abundances, size

matching can be important in understanding the interaction patterns in plant-pollinator networks. However, other causal factors need to be addressed in shaping these networks, for example, the energetic value of individual flowers, energetic requirements of pollinators and costs of resource extraction as a function of the match or mismatch between flower and pollinator size (Inouye, 1980; Smith *et al.*, 1996; Stang *et al.*, 2009).

Floral functional traits

Most plants must encourage potential pollinators to visit their flowers through features of individual flowers, whole plants or groups of plants. Plants, from a reproductive point of view, need to transfer their pollen successfully, either biotically (visitors) or abiotically (e.g. wind). Therefore, biotic pollinated plants need a visitor which is cheap to attract or feed and moving quickly to a conspecific plant. To attract such a visitor it can adapt these floral features. However, such adaptive differentiation through selection pressures is often not that clear-cut (see previous section), partially due to the fact that pollinators are mainly interested in resources and not aesthetically satisfying flowers. They do, however, associate certain floral traits with their rewards and thus this trait variation could be seen as a by-product from several factors (Ollerton, 2021).

Pollination ecology is able to provide us with insights in the evolution, ecology and, learning and foraging behaviour of plants and its pollinators (Willmer, 2011). Within this dissertation, I specifically look at the role of floral functional traits in shaping the interactions between plants and its visitors, and thus indirectly also look at such evolutionary adaptational processes. Examples of these traits are: shape, symmetry, size, floral tube dimensions and colour. Irwin, Adler & Brody (2004) categorise floral traits into two groups: traits attracting and deterring potential visitors, whereas Fenster *et al.,* (2004) made a further distinction into four groups: rewards, colour, fragrance and morphology. Pollinators must learn to associate

these floral traits with levels of rewards (Gumbert, 2000; Jersáková, Johnson, & Kindlmann, 2006; Jersáková *et al.*, 2012), which sometimes is abused by deceptive plants offering no resources (Jersáková *et al.*, 2012). Floral traits such as colour, shape, nectar or nutritious rewards and scent are seen as adaptations of flowers for efficient pollination (Junker & Blüthgen, 2010), whereas deterring traits can include e.g. floral scents, colour and unpalatable nectar. For example, potential pollinators may be attracted by floral scent compounds, while contrastingly facultative and antagonistic visitors could be repelled by the same olfactory cues (Junker & Blüthgen, 2010).

Fig. 1: A flower of *Hypoxis camerooniana.* On the left side a normal photograph, i.e. as perceived by the human eye, and on the right side an UV photograph, showing that pollinators perceive the colours differently (See **Chapter VI** on UV reflection in *H. camerooniana*). Pictures by Š. Janeček, combined by J.E.J. Mertens.

Visual cues, such as floral colour (petals, sepals and reproductive organs) and nectar guides, both visible and invisible to the human eye (i.e. infrared and UV; Fig. 1), are important in attracting potential pollinators (e.g. Peter & Johnson, 2008; Campbell *et al.*, 2010; Papiorek *et al.*, 2016; Klomberg *et al.*, 2019). Floral colour has been mentioned as an important attracting trait, with e.g. birds having a preference for red flowers (Rodríguez-Gironés & Santamaría, 2004). However, this does not mean it is exclusively reserved and while it was previously thought that red colour was an exclusion mechanism for bees, it has now been shown that this is not necessarily the case (Giurfa *et al.*, 1995; Chittka & Waser, 1997; Briscoe & Chittka, 2001; Wester *et al.*, 2020) and that its role can even differ spatially (Chen *et al.*, 2020).

Dalrymple *et al.,* (2020) show on a broad scale that both biotic and abiotic conditions are important in explaining floral colour variation. Besides insect and plant community

diversity, both mean annual precipitation and solar radiation have a high predictive power for flower colouration. Seasonality, and its related variation in food resources, is one of the most important factors determining composition, diversity and availability of pollinators is seasonality (González *et al.*, 2009; Hawkins & Devries, 2009; Albrecht *et al.*, 2018; Vandelook *et al.*, 2019; Mayr *et al.*, 2020; Classen *et al.*, 2020) which causes selection pressures. These seasonal variations in interactions can be found in the majority of tropical ecosystems (Richards *et al.*, 1996) and are also apparent in numerous partial network studies within the tropics for example for wasps (Rezende Diniz & Kitayama, 1998), butterflies (Orr & Haeuser, 1996), hummingbirds (Arriaga, Rodriguez-Estrella, & Ortega-Rubio, 1990), and sunbirds (Janeček, Bartoš, & Njabo, 2015). However, little is known about seasonal and spatial changes within complex pollination networks in the tropics (Abrahamczyk *et al.*, 2011; Souza *et al.*, 2018). A greater understanding of the role of seasonality within these plant-pollinator interactions will help not only to improve our knowledge on past and present-day processes but also to better predict the influence of climatic changes on plant-pollinator interactions.

Climate change will affect pollinators by increasing climatic seasonality and their food plants could undergo changes in distribution and phenology (Potts *et al.*, 2010), causing potential mismatches. Such changes may result in massive disruptions in highly specialised systems where one or few plant species fulfil a keystone role (Tylianakis *et al.*, 2008; Abrahamczyk *et al.*, 2011; Biella *et al.*, 2017, 2019). Recently, awareness has been raised about the decline of pollinators in many ecosystems, which could lead to widespread pollen limitation (i.e. limiting seed production by deposition of pollen grains). Commonly, pollen limitation is found when plants are self-pollinated, pollinators are scarce or deposit incompatible pollen (Fernández *et al.*, 2012; Chen & Zuo, 2019). Above mentioned ecological perturbations may lead to unusually high pollen limitation by disrupting coevolved interactions between plants and their pollinators. Since floral phenotype (i.e. observed traits) can affect pollen receipt,

selection pressures for certain floral traits could be stronger in pollen limited populations, especially concerning attractive floral traits that enhance the reliability of pollinator visits (Johnston, 1991; Wilson *et al.*, 1994; Ashman & Diefenderfer, 2001; Ashman *et al.*, 2004; Knight *et al.*, 2005; Caruso *et al.*, 2019).

Current floral trait research is still mainly focusing on both disentangling the role of individual floral traits in plant-pollinator interactions and trait combinations in an effort to validate the pollination syndrome hypothesis. Whereas countless pollination systems, including their traits have not been studied yet, especially in the understudied tropical regions of Africa and Asia (Vizentin-Bugoni *et al.*, 2018). Further research is still needed to truly understand the potentially shifting roles of countless floral traits, especially with the current development of new methods to study these traits.

Pollination syndrome hypothesis

Numerous scientists (Darwin, 1862; Müller, 1883; Knuth, 1906; Faegri & van der Pijl, 1979; Johnson & Steiner, 2000; Fenster *et al.*, 2004) underlined the link between floral trait combinations and pollinator type. Suggesting that different pollinators promote selection for diverse floral forms, resulting in the definition of pollination syndromes (Delpino, 1874; Vogel, 1954; Faegri & van der Pijl, 1979). An important underlying aspect of pollination syndromes is the concept that pollinators are clustered into functional groups that exert similar selection pressures and behave similar on a flower, which in turn created these relationships among floral traits (Waser *et al.*, 1996; Fenster *et al.*, 2004). Fenster *et al.,* (2004) defined a pollination syndrome as "a suite of convergent floral traits, associated with the attraction and utilisation of a specific group of animals as pollinators". There were initially nine pollination syndromes described around the 1960's by Vogel (1954) and Faegri & van der Pijl (1979): Cantharophily (beetles), Myophily (flies), Sapromyophily (carrion and dung flies), Psychophily (butterflies),

Sphingophily (hawkmoths), Phalaenophily (most other moths), Melittophily (bees), Ornithophily (birds) and Chiropterophily (bats). The tables listing floral traits associated to these syndromes normally including traits such as timing of anthesis, colour, nectar guides, scent, shape, nectar site/volume/concentration, pollen amount and pollen deposition site (Willmer, 2011).

Recently, several works (Santamaría & Rodríguez-Gironés, 2007; Rosas-Guerrero *et al.*, 2014; Ashworth *et al.*, 2015; Fenster *et al.*, 2015; Abrahamczyk *et al.*, 2017; Vandelook *et al.*, 2019; Dellinger, 2020) highlight the utility of syndromes for predicting or classifying species interactions. However, based on Robertson's (1928) observational data which shows that many flowers are visited by numerous animal species, several scientists questioned the accuracy of pollination syndromes in reflecting and predicting convergent selection pressures on floral traits (Waser *et al.*, 1996; Ollerton, 1996; Blüthgen *et al.*, 2007; Ollerton *et al.*, 2009, but see Fenster *et al.,* 2004). Floral antagonists exert negative selection pressures on floral traits, potentially counteracting pollinator mediated selection pressures (Gélvez-Zúñiga *et al.*, 2018).

Nevertheless, instead of rejecting or promoting the validity of the theory research has shown that the applicability of traditional syndromes (following syndromes described by Faegri & van der Pijl, 1979) falls between approximately 30% and 75% of angiosperm species (Ollerton *et al.*, 2015). Furthermore, the inclusion of both categorical and quantitative traits could be more reliable in testing the role of floral traits (Junker *et al.*, 2013; Abrahamczyk *et al.*, 2017), together with the addition of new trait expressions of flowers and visitor identities (Ollerton *et al.*, 2015; Dellinger, 2020). Additionally, applying a minimalist approach in studying plant-pollinator interactions by only using a single or few traits which matter most has been proposed as well and in some cases successful in explaining interactions (Stang *et al.*, 2006, 2007; Santamaría & Rodríguez-Gironés, 2007).

Spatial and temporal variation in the role of floral traits and its related selection pressures, which affects our ability to properly identify the role and predictive ability of floral traits has to be considered as well (González *et al.*, 2009; Abrahamczyk *et al.*, 2011; Albrecht *et al.*, 2018; Cuartas-Hernández *et al.*, 2019). In **chapter I** we give insights in the spatiotemporal variation in floral traits and its relation to plant-pollinator interactions.

The floral trait combinations part of the different syndromes cannot be considered as a matrix with each trait equally contributing to the different potential pollinator groups. This makes it hard to test pollination syndromes. Furthermore, the role of a single trait can alternate roles at different taxonomic scales and between taxa. For example, floral reward may be the most important component of shifts between e.g. functional groups of bees, while other floral traits may be more important at higher taxonomic scales (Simpson & Neff, 1983; Armbruster, 1984; Fenster *et al.*, 2004; Willmer, 2011). This in turn could result in the validity of pollination syndromes becoming taxa dependant (Krakos & Austin, 2020; Dellinger, 2020), as Krakos and Austin (2020) find the syndrome concept in *Oenothera* not being supported for most groups of visitors (only moths). They suggest a link between ancestral *Oenothera* moth pollination and current syndrome prediction accuracy (Krakos & Austin, 2020). Highlighting the need to investigate taxa specific changes and validity, as well as the inclusion of evolutionary history.

Additionally, multiple traits which are not considered in traditional pollination syndromes should be studied to expand our knowledge on the mechanisms of floral evolution (Ollerton *et al.*, 2015; Dellinger, 2020), for example pollen expulsion mechanism, an important trait related to the newly defined buzz-bee syndrome (Dellinger *et al.*, 2019). There is still work needed to fully comprehend the role of all floral traits in shaping plant-pollinator interactions and thus we rather looked at commonly recorded floral traits, to determine their individual role and as part of the syndromes.

Our research sites

It has been emphasised that limited data on plant-pollinator networks from the tropics exists, which host the most complex and species rich communities and represent natural evolutionary laboratories (Schleuning *et al.*, 2012; Trøjelsgaard & Olesen, 2013; Vizentin-Bugoni *et al.*, 2018). Data from these areas are important in understanding the ecology and evolution of complex plant-pollinator networks (Trøjelsgaard & Olesen, 2013). The diversity of flowering plants and their pollinators is remarkably higher in the tropics compared to temperate regions (Terborgh, 1992). Therefore, the prevalent expectation among pollination biologists has been that tropical regions should host more specialised interactions and complex communities (MacArthur, 1972). However, as discussed above, inconsistent results have been found (Schleuning *et al.*, 2012; Trøjelsgaard & Olesen, 2013; Pauw & Stanway, 2015). Dalsgaard *et al.,* (2011) showed that biotic specialisation is strongly linked to species-rich networks and that tropical plant-hummingbird networks are more specialised than sub-tropical and temperate counterparts. Several studies found no such patterns when comparing plant-pollinator interactions from different bioregions (Ollerton & Cranmer, 2002; Olesen & Jordano, 2002). These uncertainties show that we are still in the process of understanding pollination networks worldwide. Ollerton (2012) stipulates that the lack of tropical studies could have influenced the findings of Schleuning *et al.,* (2012) and calls for more research on the understudied Afrotropical and Asian pollination systems to gain a better understanding of network characteristics worldwide. Furthermore, he shows that the sensitivity to perturbation is geographically complex rather than predictable from latitude alone.

In Africa pollination research has been primarily focused on the subtropical and temperate southern Africa, although there are some noteworthy exceptions of partial network studies by my collaborators involved (Bartoš *et al.*, 2012; Janeček *et al.*, 2012, 2015; Padyšáková *et al.*, 2013; Bartoš & Janeček, 2014; Vlašánková *et al.*, 2017; Mertens *et al.*, 2018,

2020) and comprehensive studies done on Mount Kilimanjaro (e.g. Albrecht *et al.,* 2018; Mayr *et al.,* 2020; Classen *et al.,* 2020). Nevertheless, northern hemisphere studies are more common, although they deal with less diversity in both plant and pollinator communities.

To help gain a better understanding of plant-pollinator interactions and how floral traits influence them in the Afrotropics we focused on Mount Cameroon (4.203°N, 9.170°E; **Chapters I–III & VI**),). Mount Cameroon is located in the Southwest Region of Cameroon and is the highest mountain in western and central sub-Saharan Africa. Due to its location in the Gulf of Guinea Highlands and bordering the Congolese and Guinean bioregions it offers a high species diversity and endemism in a wide range of habitats (Cheek *et al.*, 1996; Cable & Cheek, 1998; Maicher *et al.*, 2018). Mount Cameroon is unique since it still hosts a continuous gradient from pristine lowland rainforest up to montane forests at the timberline on its southern flanks. Therefore, we studied the plant-pollinator interactions at four elevations along this gradient (Fig 2). The region is also known for a strong seasonality with monthly precipitation being over 2000mm at lowlands on the seashore in the wet season (June to September) and almost no rain in the dry season (December to March). We included both extremities (dry and wet) in our sampling (Maicher *et al.*, 2018, 2020).

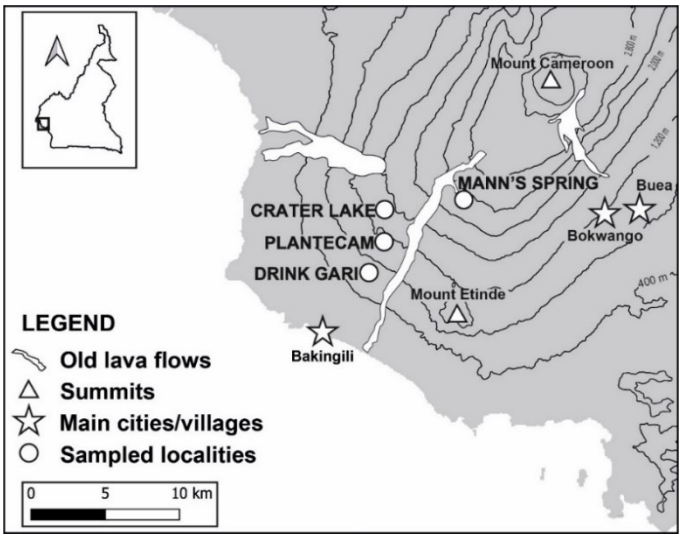


Fig. 2: Map of our study area on Mount Cameroon, adapted from Ustjuzhanin *et al.,* (2020).

Another region for which we are lacking knowledge in plant-pollinator interactions and pollen limitation studies was found in Central and Eastern Europe (Bennett *et al.*, 2018; Bartoš *et al.*, 2020). According to Pain & Travis (2009) and Boakes *et al.*, (2010) these knowledge gaps were connected to the ex-communist governments' tight controls on science funding and a low priority of conservation-related research (Pain & Travis, 2009; Boakes *et al.*, 2010). Our studies (**Chapters IV & V**) have helped in gaining more knowledge on the role of plant-pollinator interactions in Central European meadows in relation to both plant mating systems and pollen limitation. During the 2016 and 2017 vegetation seasons we studied plants in the semi-natural wet meadows of the Železné Hory Protected Landscape area. This is part of the Bohemian-Moravian highlands in the Czech Republic. Habitat fragmentation due to anthropogenic influences resulted in these meadows being mere remnants of formerly common wetland systems, which were maintained by extensive agriculture in the past (Janeček *et al.*, 2013). In **Chapter IV** we collected 46 plant species from these sites for our exclusion experiments in the greenhouse at the Institute of Botany of the Czech Academy of Sciences in Třeboň, whereas in **Chapter V** we studied pollen limitation on a specific meadow near the Chobotovský rybník pond (49°46'57"N, 15°50'17"E; Fig. 3). Further works on these beautiful meadows are not part of this dissertation.

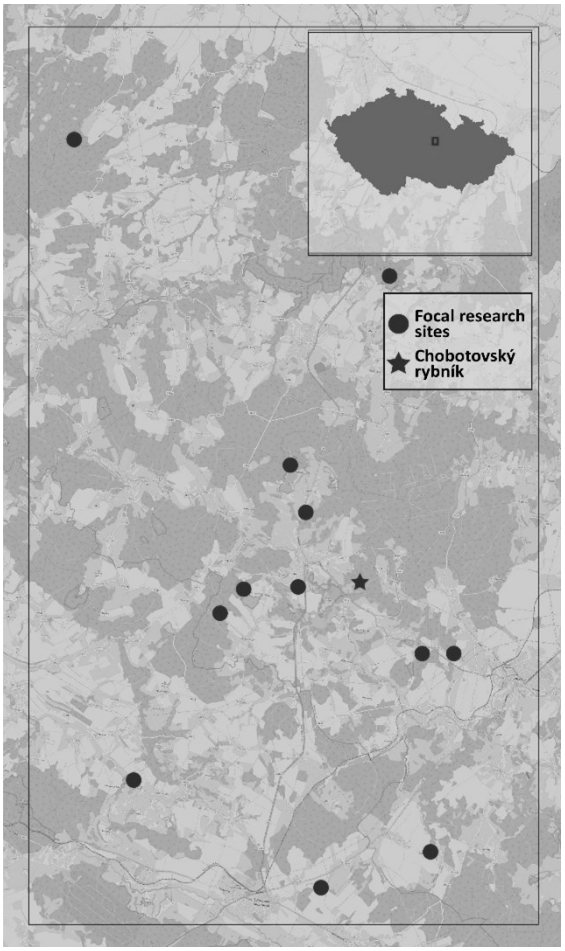


Fig. 3: Map of Železné Hory with our focal research sites including Chobotovský rybník

OUTLINE OF DISSERTATION

Aims

In this dissertation, the main goal was to further investigate the role of selected plant floral traits in plant-pollinator interactions. Initially, I aimed to mainly question the validity of syndromes, since large-scaled syntheses showed inconclusive evidence and there was a data gap in pollination syndrome research, particularly in the Afrotropics and Central/Eastern Europe (Ollerton *et al.*, 2009; Rosas-Guerrero *et al.*, 2014; Ashworth *et al.*, 2015; Vizentin-Bugoni *et al.*, 2018). Especially in specialised systems, floral traits have been shown to play an important role in shaping the interactions and could even be linked to syndromes. However, pollination syndromes and the role of floral traits are not as simplistic as often shown. Often, we are still lacking qualitative data on basic pollination-related traits (Dellinger, 2020). Therefore, I decided to step away from syndromes and the discussion surrounding it. Which allowed me to rather disassemble the syndromes and elaborate more on the role of individual traits and their combinations in shaping plant-pollinator interactions.

Additionally, several works found evidence for changing roles of individual floral traits and related selection pressures in space and time (e.g. González *et al.*, 2009; Abrahamczyk *et al.*, 2011; Albrecht *et al.*, 2018). Therefore, I have included spatiotemporal variation in our study of floral trait distribution and importance as well.

This dissertation mainly aims to:

i) gain a better understanding of the role of floral traits in shaping and predicting plant-pollinator interactions (**Chapters I-III & VI**),

ii) investigate how the trait distribution and role shifts between season and elevations (**Chapters I & II**),

iii) investigate how reproductive traits and pollinator interactions are associated with plant mating systems and pollen limitation (**Chapters IV & V**).

Outline and outcomes

Therefore, I have tried, together with all collaborators, to give insights into the role of selected basic pollination-related traits might play in the complex plant-pollinator interactions of Mount Cameroon (**Chapters I-III & VI**). Additionally, we also studied the role of reproductive traits in shaping mating systems and pollen limitation (**Chapters IV & V**). The study of individual traits can be a lifelong obsession and cannot be dealt with properly in a single dissertation. Nevertheless, I hope the data presented here will help our understanding and be useful for further research.

The key chapter (**Chapter I**) of this dissertation focused on seasonal and elevational changes in floral trait distribution and importance. As mentioned in the introduction, uncertainties remain whether trait combinations, such as pollination syndromes, can accurately predict pollinator interactions and if following a complex predetermined list of equally important traits could not be problematic in said predictions. Nevertheless, to a high degree, this discrepancy can be caused by individual functional groups of pollinator and environmental specificity of the importance of individual floral traits. Therefore, in this chapter we aimed to: i) study shifts in trait importance among individual pollinator groups, ii) asses environmentally driven changes in trait distribution, importance and predictability of pollinators. Using Random Forest models (Breiman, 2001; Dellinger *et al.*, 2019), which assigns different significances to individual floral traits, we demonstrated that the significance of individual traits in plant-pollinator interactions differs under various environmental conditions and floral traits can predict potential pollinators relatively well. Using the most important traits (Shape, Sugar per flower, Colour, Size and Tube Length) from these Random Forest models in our Canonical Correspondence Analyses we were able to show that the distribution of the trait characteristics differs spatiotemporally, and traits were better in explaining the variation in pollinator functional groups towards harsher conditions, i.e. higher elevations and wet season. For

example, traits preventing nectar dilution and pollen washing were found more commonly in the wet season, together with red coloured flowers, which can be linked to the increased role of birds as pollinators present during that time.

Due to this uncovered spatiotemporal variation, instead of continuing focussing on questioning the validity of syndromes, I instead propose to follow Ollerton *et al.,* (2015) and Dellinger (2020), to use more flexible definitions and weights of particular traits for different syndromes and conditions. Additionally, we should also put more effort on further studying numerous commonly and uncommonly used traits and visitors. Thus, improving the trait-based understanding of plant-pollinator interactions, which in turn could benefit pollination syndrome studies.

An in-depth look at two specific pollinator groups, being i) butterflies and hawkmoths (**Chapter II**), and ii) nectarivorous birds (C**hapter III**), has given additional insights on the role of floral traits in shaping their interactions besides the general patterns found in **Chapter I**. Butterflies and hawkmoths are important pollinators and certain floral traits (e.g. corolla tube length) are known to be important in shaping their floral preferences. From these functional visitors' groups, we have collected morphometric measurements, such as proboscis length, allowing us to consider trait matching. In this study (**Chapter II**) we aimed to i) evaluate the role of both functional groups in plant-pollinator networks among elevations and between seasons, including shifts in their relative importance and specialisation, and ii) assess the floral preferences of butterflies and hawkmoths. Besides the expected link between corolla tube length and proboscis length we also expected that floral preferences revolve around flower size, colour, and tube length at the community level. Our results showed visitor species richness decreasing towards harsher environments, i.e. higher elevations and wet season. Additionally, visitation frequency varied among the lepidopteran families and between elevations and seasons. Higher frequencies were found in mid elevations and in dry season, which follows

general patterns in lepidopteran diversity. We also found a significant positive correlation between proboscis length and corolla tube length. However, at visitor family level, the observed relationship was only significant in hesperiid butterflies. Additionally, the studied functional groups showed a significant preference towards certain floral traits, with a quarter of the variability in visitation frequency explained by selected floral traits. We found three distinct groups: i) sphingids preferred sugar-rich nectar, larger and deeper flowers of purple colour; ii) papilionids, lycaenids and nymphalids preferred orange-coloured flowers, and iii) no clear preference was found for hesperiids and pierids in our model. In conclusion, we found shifts in the species richness, composition and visitation frequency along elevations and between seasons with floral traits being important in shaping visitation by butterflies and hawkmoths.

In **Chapter III**, we elaborated on sunbird pollination and specifically whether pollination syndromes are valid for birds on Mount Cameroon. Additionally, we wanted to check whether birds actually care about floral traits or just care about the nectar resources provided. We combined our main dataset (**used in Chapters I & II**) with a bird-centred dataset from the same localities on Mount Cameroon. We tested i) whether insects and birds interact with different syndrome plant species; ii) if plants with the bird pollination syndrome related floral traits interact mainly with birds; iii) if birds favour these ornithophilous plants, and iv) if and how these individual floral traits predict bird visitation. Rather than showing a strong separation into bird or insect hosting plants we found a continuum of strategies. We confirmed the validity of the bird pollination syndrome hypothesis from the plants' perspective, with ornithophilous plants visited at a higher frequency by birds and the majority of them hosting a low amount of insect visitors. However, nectar production per plant individual was a better predictor of bird visitation than any other floral trait. Showing that birds tend to not care as much about the floral hosts' traits, as long as it provides enough resources.

In all three chapters we confirmed that nectar is an important trait shaping interactions and useful in predicting potential pollinators (also highlighted in e.g. Stang *et al.*, 2006; Dellinger, 2020), however as we also showed that from the birds' perspective, this trait importance does not have to follow a certain set of trait preferences. Rather the visitors' floral choice is governed primarily by the amount and availability of nectar rewards (Pleasants & Waser, 1985; Schmid *et al.*, 2016). Floral reward (mainly nectar) is commonly used in distinguishing pollinators and syndromes (Dellinger, 2020). Both specifically studied groups (Lepidoptera and Sunbirds) are nectarivorous, therefore it is not surprising that nectar plays a big role in shaping their visitation. Pollen feeding visitors could be influenced by different selection pressures, with other traits becoming important, such as (UV) colour (See **Chapter VI**). Initial works, which applied Random Forest models, have shown that not all floral traits are equally important in each pollination syndrome and the role of uncommonly studied traits is largely unknown (Johnson, 2013; Dellinger, 2020). Therefore, caution is needed when selecting traits and trait combinations for study of pollination syndromes and trait importance, and some argue that it would be unwise to dismiss any trait from further studies yet (Fenster *et al.*, 2015; Dellinger *et al.*, 2019; Dellinger, 2020). However, in my opinion the future of trait-based studies not only lies in the inclusion of more and different traits, but also to step away from these unweighted measures and include stronger methodologies which can account for shifts in the role of certain traits for pollinator groups, such as machine learning methods (Pichler *et al.*, 2020).

Another question we tried to answer is how specific traits influence plant reproduction through mating systems of flowers and thus possibly influence the potential pollination success (**Chapters IV & V**)? Self-compatibility, and especially autonomous self-pollination, can become particularly beneficial in degraded landscapes lacking potential pollinators. In a hand-

pollination greenhouse experiment with plants from wet meadows of the Železné Hory Mountains, Czech Republic, we evaluated i) the mating systems of important flowering species and ii) the role of traits in shaping these systems. We analysed the relationships between 12 traits, both floral and life-history, and the species' mating systems (**Chapter IV**). The studied meadow species were grouped into four systems. Fully and partially self-incompatible species formed the largest group, followed by self-compatible non-selfers, and lastly a group which did not fit a single system specifically and was therefore named as the mixed mating group. We did find that nectar sugar per flower, nectar sugar per shoot, and dichogamy were significantly associated with the mating systems. Especially, non-selfers produced the largest amount of nectar, which obviously helps in attracting of pollinators. Additionally, we did not find signs of inbreeding depression in the majority of the species. This together with a low frequency of spontaneous selfers suggest the existence of a selection against selfing. Floral traits, such as the level of dichogamy and amount of nectar reward, could potentially affect the balance between selfing and outcrossing rates in the self-compatible species and thus shape the evolution of mating systems.

We also performed a field pollen supplementation experiment to study in 22 plant species in a wet semi-natural meadow: i) the level of pollen limitation in a specific wet meadow flowering plant community, and ii) the correlation between multiple functional traits of plant species, as well as pollinator foraging behaviour, with pollen limitation (**Chapter V**). We found significant pollen limitation for approximately 41% of species, which was much lower when compared to single-species studies, but higher than all other community-level studies. This outcome could be caused by some issues such as sampling or publication biases, in known research. Seven species had a significant positive response in seed production and two species increased in seed weight after pollen supplementation. Only the number of pollinator functional groups significantly affected pollen limitation, however other traits which were not included in

this study could still play a role. For example, floral size, shape and longevity have been mentioned before (Ashman *et al.*, 2004; Fenster *et al.*, 2004; Knight *et al.*, 2005). Of course, these traits have been shown to influence pollinator visitation (e.g. **Chapter I**). Even though we found that the number of pollinator functional groups to influence pollen limitation, like the findings of Knight *et al.,* (2005), questions regarding the specialisation of pollinators remain. Both increases and decreases in pollen limitation have been found. On the one hand, specialised visitors provide more reliable pollination (i.e. lower pollen limitation), whilst pollination success might decrease due to an over-abundance of generalist visitors (Waser *et al.*, 1996; Culley, Weller, & Sakai, 2002; Knight *et al.*, 2005).

Finally, to look further at the role traits play in shaping plant-pollinator interactions, we also did an experiment on the influence of an aspect of floral colour, ultraviolet (UV) colouration (**Chapter VI**). Apart from floral morphology and colours perceived by the human eye, UV reflectance acts as an important visual advertisement of numerous flowering plant species for pollinators. However, the effect of UV signalling on attracting pollinators of particular plant species is still insufficiently studied. Therefore, we studied: i) the pollination system of *Hypoxis camerooniana,* ii) the effects of the flowers' UV reflectance on its visitors by experimentally removing UV reflection from petals, and iii) the role of changing the UV pattern in visitation, by partial removal of UV.

Due to their contacts with reproductive organs, bees are considered as the primary pollinators of *H. camerooniana*. Partial removal of UV did not influence visitation significantly, whereas complete removal did decrease visitation rates and affect the landing behaviour of the bees. Thus, we conclude that it is highly probable that UV reflectance is important for localising the flowers in the mountain grasslands of Mount Cameroon, whilst the UV pattern plays a lesser role. The results mentioned above are in concurrence with other works on this topic and shows that UV reflectance, together with other factors within floral colour, is

another channel of communication of plants with insects, which requires our attention in fully understanding the interplay between plants and its visitors (Chittka *et al.*, 1994; Johnson & Andersson, 2002; Peter & Johnson, 2008; Koski & Ashman, 2014). Additionally, we also tested whether our experimental controls where a good reflection of the natural situation (natural control) and found that our experimental approach influenced visitation frequencies regardless of treatment type. We recommended future studies with experimental manipulations of floral (UV) colour and patterns to include untouched natural controls.

Conclusions

While I expected the role of floral traits to be of an increased importance in the more specialised networks, i.e. the species rich lower elevations, I found that the importance of traits in shaping interactions increasing towards harsher environments (**Chapter I**). Moreover, changes in floral trait importance for functional groups of pollinators have been shown (**Chapters I-III & VI**). Therefore, I conclude that traditional pollination syndromes, although being good principles, should rather be disentangled and studies should first focus on the (shifting) roles of individual traits. Random Forest models could help in this, as it has proven to be relatively successful in predicting interactions of pollinator groups without following the traditional pollination syndrome approach. With increased availability of stronger computational power, we will also see better methods for analyses (e.g. Pichler *et al.*, 2020). However, for larger-scale analyses, I think we still need to unify our floral trait sampling methodology in order to give insights into patterns of trait prediction or importance.

Even though we did confirm the validity of the pollination syndrome hypothesis for bird visited plants (and to some extent for butterfly and hawkmoth plants as well), birds rather focussed on resource availability and tended not to care as much about other traits. **Chapter I-IV** emphasize the importance of nectar in shaping interactions and even mating systems. Our

unpublished data (Janeček *et al.,* unpublished) on nectar trait properties in relation to visitation by pollinators dove deeper into these carbohydrate traits. Showing that not all groups of plants with the same primary pollinator have typical nectar characteristics. Although we did find differences between insect-pollinated and bird-pollinated plants, with ornithophilous plants producing larger amounts of sugar with a high proportion of sucrose. This, together with our other findings, such as the increased importance of UV in bees, again highlights the need to look at the broader, and taxa specific, roles of specific floral traits in shaping interactions, prior to evaluating pollination syndromes (as mentioned by e.g. Krakos & Austin, 2020; Dellinger, 2020). The study of floral traits is still evolving, and it will take time to fully understand the role of all traits, if we ever will? Nevertheless, I did shed some light on the role of several pollination related traits in an understudied region, which can help as a basis for further work. For example, besides above-mentioned nectar study, we also have data on spectrophotometry and UV photographs available, which can be the basis for more specific floral colour-related research on Mount Cameroon.

In conclusion, my dissertation proved the variability in the importance of floral traits in shaping not only plant-pollinator interactions but also mating systems. These pollinator groups can in turn influence pollen limitation, resulting in complex, mutualistic, systems which are in need of further studies worldwide to fully comprehend the occurring interplay. With increased global pressure on our pollinators, it is even more important to understand these systems fully, especially since pollinators play an important role in ecosystems. With the manuscripts and published papers in this dissertation, I do give some insights from some understudied systems. Nevertheless, still further work can, should and will be done in the near future to improve our trait-based understanding.

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CHAPTER I

Klomberg Y, Tropek R, Mertens JEJ, Kobe IN, Hodeček J, Raška J, Fominka NT, Souto-Vilarós D, Janeček Š (Manuscript). Spatiotemporal shifts in the role of floral traits in shaping tropical plant-pollinator interactions. *Published as preprint in BioRxiv: https://doi.org/10.1101/2020.10.16.342386*

YK was highly involved in the conceptualisation of this study, sampling of field data and their processing; he was in charge of all analyses, and their interpretations. First versions of this manuscript were written by YK under the supervision of ŠJ.

Spatiotemporal shifts in the role of floral traits in shaping tropical plant-pollinator interactions

Running title: Shifting role of floral traits for pollinators

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Author Contribution: RT, ŠJ and YK conceptualised this study. YK, RT, JEJM, INK, NTF and ŠJ participated in fieldwork. RT, ŠJ, JEJM and YK were in charge of data processing, whilst JH and JR identified the insect visitors. Analyses were done by YK with significant input from RT, ŠJ, JEJM and DSV. A first version was written by YK under the supervision of ŠJ, all authors contributed to writing and editing of the manuscript.

Data and code availability: Upon acceptance of the manuscript, data used for analyses will be made available through an appropriate public repository, such as Zenodo, and the data DOI will be included. Code is available through our **Github repository**.

Keywords: Afrotropics, Foraging behaviour, Mount Cameroon National Park, Pollination syndrome, Pollination systems, Pollinator predictability

Abstract

The pollination syndrome hypothesis predicts that plants pollinated by the same pollinator group bear convergent combinations of specific floral functional traits. Nevertheless, some studies have shown relatively low predictive power for these floral trait combinations. This discrepancy may be caused by changes in the importance of specific floral traits for shaping interactions under different environmental conditions and for different pollinator groups. To test this, we studied pollination systems and floral traits along an elevational gradient on Mount Cameroon during wet and dry seasons. Using Random Forest models, allowing the ranking of traits by significance, we demonstrated that some floral traits are more important than others in shaping interactions and that these traits predict pollinators relatively well. However, the distribution and importance of traits varies under different environmental conditions. Our results imply the need to improve our trait-based understanding of plant-pollinator interactions to better inform the debate surrounding pollination syndrome hypothesis.

Introduction

The importance of floral traits for plant-pollinator interactions has been apparent since the 18th century (Sprengel, 1793; Müller, 1883; Knuth, 1906). Darwin placed the origin of floral traits into the modern evolutionary framework (Darwin, 1859, 1862) and during the 19th and 20th century other scholars followed by suggesting various floral trait classifications according to their adaptive relationships to particular pollinator groups. These efforts resulted in an influential ecological and evolutionary hypothesis, the pollination syndrome hypothesis (Delpino, 1874; Vogel, 1954; Faegri & van der Pijl, 1979; Fenster *et al.*, 2004; Waser, 2006). It is defined as a set of convergent floral traits (e.g. colour, shape, odour or production and display of floral rewards) evolved to attract a particular group of pollinators (Faegri & van der Pijl, 1979).

Despite such a long research history, the pollination syndrome hypothesis has been questioned in recent decades. One of the main reasons is that community wide studies exploring complex plant-pollinator networks demonstrated a higher level of generalisation in pollination systems and lower predictability of pollinators based on floral traits than previously expected (Waser *et al.*, 1996; Johnson & Steiner, 2000; Ollerton *et al.*, 2009). Recent empirical efforts have provided evidence both supporting (e.g. Hargreaves *et al.* 2004; Rosas-Guerrero *et al.* 2014; Fenster *et al.* 2015; Dellinger *et al.* 2019a; Vandelook *et al.* 2019) and contradicting (e.g. Blüthgen *et al.* 2007; Ollerton *et al.* 2009; Paudel *et al.* 2019; Rocha *et al.* 2020; Wang *et al.* 2020) the validity of the pollination syndrome hypothesis. Researchers also demonstrated that pollination systems can show parallel adaptations to multiple pollinator groups (Dellinger *et al.*, 2019b), and thus suggested reclassification of some pollination syndromes (Dellinger *et al.*, 2019a), or proposed that pollination syndrome theory can be improved by other concepts, like optimal foraging theory or evolution stable strategy (Pyke, 2016). Additionally, Abrahamczyk *et al.* (2017) highlight the importance of using quantitative data, besides categorical traits, in testing the efficiency of pollination syndromes.

At the same time detailed studies on individual traits included within pollination syndromes have shown that we do not fully understand their functionality and importance (Dellinger, 2020). For instance, pollination and use of long spurred flowers does not necessarily correspond to long-proboscid visitors (Vlašánková *et al.*, 2017), or that hummingbird visits are driven by nectar reward rather than other floral traits (Maruyama *et al.*, 2013). Furthermore, the observed trait configuration does not need to be an adaptation to pollinators alone. Floral antagonists exert negative selection pressures on floral traits, like flower size or the number of flowers on a plant, counteracting pollinator mediated selection pressures (Gélvez-Zúñiga *et al.*, 2018). For example, it is still questionable to which extent red flowers are an adaptation to bird vision, or a defence against nectar thieving bees (Chittka & Waser, 1997; Rodríguez-Gironés

& Santamaría, 2004; Wester *et al.*, 2020) and if this role shifts spatially (Chen *et al.*, 2020). Moreover, from a methodological point of view, if we acknowledge that individual floral traits largely differ in their role in shaping plant-pollinator interactions (e.g. Maruyama *et al.* 2013; Schmid *et al.* 2015), and that their synergistic effects are important (Fenster *et al.*, 2015), then the original category-based ordinations and classifications of individual traits do not seem to be the best expression of the real situation in nature (Abrahamczyk *et al.* 2017; Fig. **1a,b**). Hence, new methods are needed to reflect more complex interactions among predictors (i.e. floral traits) and to assess their importance for pollinators (Cutler *et al.*, 2007; Johnson, 2013; Dellinger *et al.*, 2019a; Pichler *et al.*, 2020).

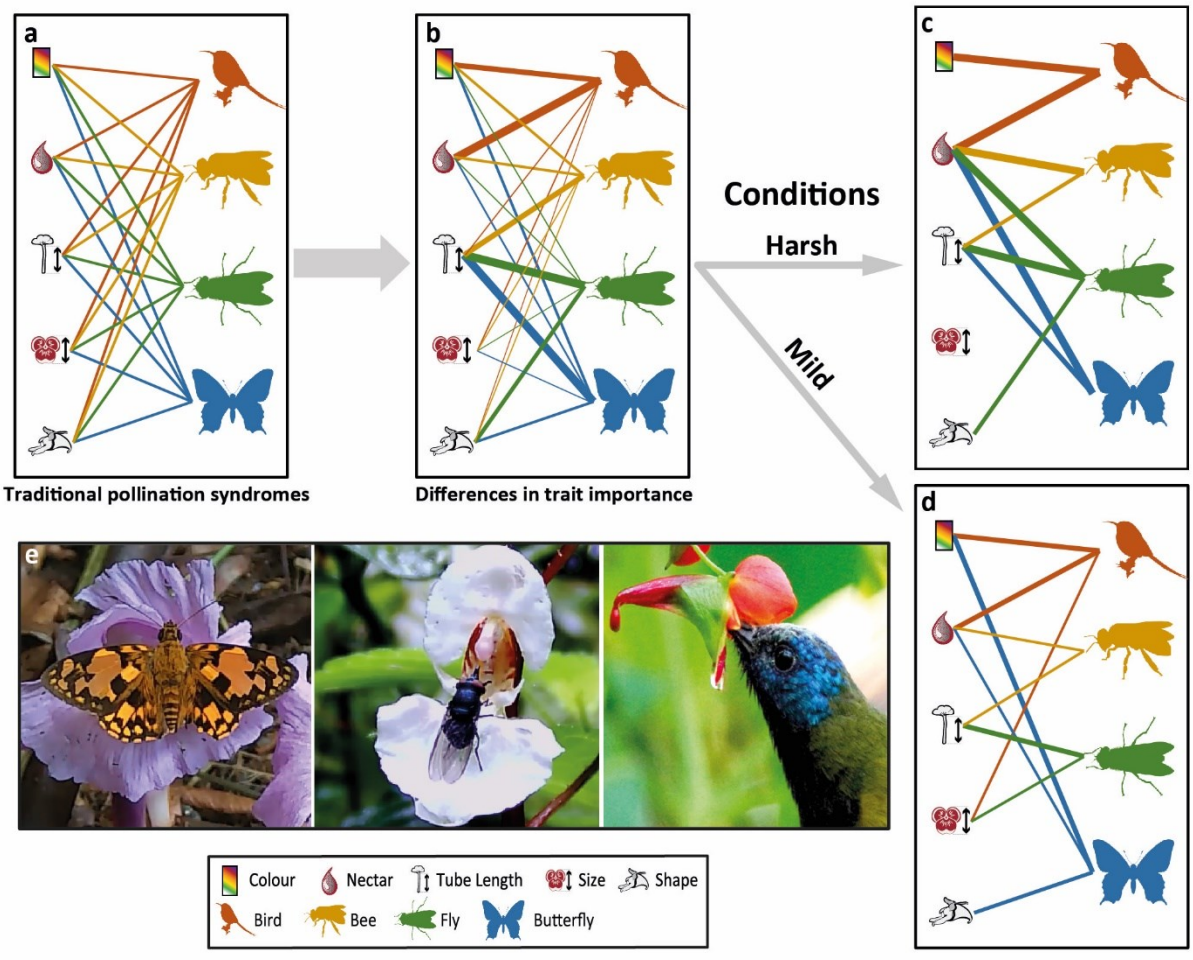

Fig. 1. Conceptual figure. a) showing the traditional pollination syndrome view on the affiliation of plant traits and individual pollinator groups. b) floral traits differ in their relative importance for individual pollinator groups. c and d) The relative importance of individual floral traits differs between harsh and

mild conditions: along the elevational gradient and between seasons. e) Examples of flower visitors found on Mount Cameroon: *Apallaga meditrina* on *Aframomum sp.*, *Rhingia sp.* on *Impatiens burtonii* and *Cyanomitra oritis* on *Impatiens sakeriana* (First two pictures are screenshots from the video recordings, the last picture was made by Š. Janeček). Note that relationships shown here do not reflect the real situation and are purely meant to conceptualise the hypotheses.

The second problem is that the role of individual floral traits and the related selection pressures can vary in space and time (González *et al.* 2009; Hawkins & Devries, 2009; Abrahamczyk *et al.* 2011; Albrecht *et al.* 2018; Mayr *et al.* 2020; Mertens *et al.* 2020; Fig. **1c,d**), which can make the formation of general conclusions regarding the ability of traits to predict pollinators even more challenging. However, understanding these spatiotemporal patterns can be crucial for revealing the role of floral traits in shaping plant-pollinator interactions.

Elevational gradients in seasonal ecosystems feature high adaptive trait differentiation and are thus an ideal place to study possible spatiotemporal variability in the relative importance of individual floral traits. Elevational gradients allow us to observe substantial changes in abiotic and biotic conditions (McCain & Grytnes, 2010; Girardin *et al.*, 2014), including changes in taxonomical and functional diversities of plants and pollinators (Janeček, Bartoš, & Njabo, 2015; Albrecht *et al.*, 2018; Cuartas-Hernández *et al.*, 2019), as well as their interactions (Olesen & Jordano, 2002; Ramos-Jiliberto *et al.*, 2010). Shifts in occurrence and/or abundances of individual floral traits have also been reported; their role in shaping biodiversity, however, remains unclear (Sun, Gross, & Schiestl, 2013). In addition, these elevational patterns can also differ seasonally (Maicher *et al.*, 2020).

The importance of individual floral traits can be related to pollinator requirements and pollinator-community organisation. At higher elevations, pollinators have greater energetic requirements due to lower temperatures (Classen *et al.*, 2015) or lower air pressure which hinders flight (Feinsinger *et al.*, 1979). In turn, this can increase the importance of floral traits

related to rewards (e.g. nectar production or concentrations). Nectar production can increase in importance under less suitable seasonal conditions, such as during the wet season in humid tropical forests (Cruden, 1972; Janeček *et al.*, 2015). This might be connected with the increasing prevalence of larger pollinators during wet seasons, such as nectarivorous birds, whose flight is less effected by rainy conditions compared to insects (Cruden, 1972). In contrast, Robinson & Wilson (1998) argued that according to the optimal foraging theory, increased resource availability could lead to more niche partitioning, and thus potentially better pollinator predictability by floral traits. We found increased resource availability at lower elevations (unpublished results), thus traits related to floral advertisement (e.g. colour or scent) could potentially be more important there. In seasons with less resources we can expect higher specialisation (Souza *et al.*, 2018) and consequently, an increased ability to predict pollinators using floral traits.

To reveal the spatiotemporal variability in predictive power and relative importance of individual floral traits, data were collected on plant-pollinator interactions and floral traits on the community level, at four rainforest elevations along an elevational gradient on the highest West African mountain, Mount Cameroon. Data was sampled in distinct wet and dry seasons, with extreme rains in the wet season (>1,500 mm monthly) and almost no rain during the dry season (Maicher *et al.*, 2020). The following hypotheses were tested:

1) The relative importance of individual floral traits differs among individual pollinator groups (Fig. **1b**). 2) There are environmentally driven changes in trait distribution and importance: 2.1) There is differentiation within floral trait distribution along the elevational gradient and between seasons. 2.2) Changes in the ability of traits to predict pollinators are found in harsh versus mild environmental conditions, i.e. along the elevational gradient and between seasons (Fig. **1c,d**).

Materials and Methods

Study locality

This study was carried out on Mount Cameroon, Southwest Region of Cameroon (4.203°N and 9.170°E), the highest mountain in western and central sub-Saharan Africa (4,095 m a.s.l.; Cable & Cheek, 1998). It represents an important biodiversity and endemism hotspot due to its location in the Cameroon Volcanic line (Gulf of Guinea Highlands) on the border of the Congo and Guinean bioregions, offering a wide range of habitats (Cable & Cheek, 1998; Sosef *et al.*, 2017; Maicher *et al.*, 2018, 2020).

We focused on the continuous elevational gradient of pristine rainforests, from lowland (±650 m a.s.l.), sub-montane (±1,100 and ± 1,450 m a.s.l.) to montane (± 2,200 m a.s.l.) forests at the natural timberline on the southwestern slope of the mountain (For more details see Maicher *et al.* 2020). The region is known for its strong seasonality with annual precipitation exceeding 12,000 mm at the lower elevations in proximity to the Atlantic ocean with monthly precipitation in the wet season (June to September) of over 2,000 mm and almost no rainfall during the dry season (Maicher *et al.*, 2018, 2020). At each studied elevation, six transects of 200m long and 10m wide (5m on both sides of the transect-line) were established at least 100m apart to cover the heterogeneity of the forest. Data sampling was performed along these transects; however, the search area also included the surrounding vegetation when there were insufficient replicates for a particular plant species. Sampling took place in the dry and wet season of 2017 (1,450 and 2,200m a.s.l) and 2018 (650 and 1,100m a.s.l).

Floral trait measurements

Following Ollerton and Watts (2000) and Ollerton *et al.* (2009) we selected floral traits important for potential pollinators (Table **S1**). Morphometric traits (Size, Tube length/width) were measured by an electronic calliper, visual (shape, symmetry, colour, flower/anther

position and presence of nectar guides) and olfactory traits (strength of scent) were recorded by the observer (Table **S1**). Initially scent type was also included following the classification by Ollerton *et al.* (2009), however due to potential observer bias within this trait we chose to exclude it and keep the simplified classification of weak to no smell and a strong smell. Additionally, to minimize observer bias in other recorded traits, such as shape and flower position, all plant species were checked afterwards, and traits confirmed by two botanists. Up to five individuals of 121 plant species were measured.

Quantification of nectar sugar production was done by covering flowers with mesh bags for a 24-hour period. Nectar from individual flowers with high nectar production was extracted following Bartoš *et al.* (2012) using capillary tubes. The nectar concentration was measured using a Pal-1 (Atago co.) pocket refractometer, after which we were able to calculate the amount of sugar per µl of nectar: $y = 0.00226 + (0.00937\ x) + (0.0000585\ x^2)$, where x is the nectar concentration and y is mg of sugar per µl (Galetto & Bernardello, 2005). Using the measured volume in capillary tubes we converted y to amount of sugar per flower. For low nectar-producing flowers, we washed the flowers using a Hamilton syringe with filtered water, added ethanol to the samples and boiled it for 15 minutes to deactivate enzymes. Nectar samples were dried in the laboratory, where they were transferred into constant volumes. The concentrations of individual sugars were measured with High-Performance Liquid Chromatography (HPLC) using the ICS-3000 system (Dionex) with an electrochemical detector and CarboPac PA 1 column. Our main dataset includes more than 121 plant species, but we excluded species that lacked complete floral trait measurements (morphometric or nectar) from our analyses.

Visitor recording

The flowering plant species along the set transects were recorded for a 24-hour period using security cameras (VIVOTEK IB8367T with IR night vision; for more information on the

methodology see Mertens *et al.* 2018, 2020; Klomberg *et al.* 2019) to detect and identify flower visitors to 13 functional groups of pollinators. We recorded flowers at all vegetation strata from understory to canopies (reached using tree climbing methods).

The functional groups were defined following the common pollination syndrome groups (Birds, Bats, Flies, Bees, Wasps, Butterflies, Hawkmoths, Other moths, Non-flying-mammals; Willmer 2011). Bees were split into Honeybees, Carpenter bees (as the large representative of the recently defined "buzz pollination syndrome"; De Luca & Vallejo-Marín, 2013) and Other bees to better reflect floral reward usage. Flies were split into Hoverflies and Other flies. Cockroaches were also considered as potential pollinators (Mertens *et al.*, 2018; Vlasáková *et al.*, 2019; Xiong *et al.*, 2020). Only visitors seen touching plant reproductive organs (anthers or stigmas) were considered as potential pollinators (called pollinators elsewhere in our paper) and included in our analyses (Padyšáková *et al.*, 2013). The concept of most effective pollinator has been treated elsewhere as the product of visitation frequency or pollen transfer efficiency (e.g. Rosas-Guerrero *et al.* 2014; Ashworth *et al.* 2015; Fenster *et al.* 2015). However, due to the scale of this work we were not able to classify pollen transfer efficiency and therefore relied on contacts with reproductive organs as a proxy of pollination (similar to e.g. Biella *et al.* 2019).

Statistical analyses

Following Dellinger *et al.* (2019a) we used Random Forest models (RF; Breiman, 2001) to identify the most important floral traits differentiating potential primary and secondary pollinators. Secondary pollinators were included to account for the possibility that the most frequent visitor does not have to be equivalent to the most effective pollinator (Mayfield, 2001; Padyšáková *et al.*, 2013; Ashworth *et al.*, 2015; Barrios *et al.*, 2016). RF is a classifier tool capable of modelling complex interactions among both categorical and continuous predictor variables. In RF analyses a specified number of decision trees are built based on subsets of the

data and permutated generally 100 times. At each node different variables are tested and the quality of a specific variable in reducing the model's entropy is measured (Gini index). Since only part of the input data is used in each tree we can estimate classification error and model accuracy reduction when a single character is removed (Cutler *et al.* 2007; Johnson 2013; Dellinger *et al.* 2019a). Following several concerns (Strobl *et al.*, 2008; Parr *et al.*, 2018; Scheidel, 2018), we looked at the mean decrease in accuracy without scaling, due to mentioned inaccuracies related to the Gini Index. Therefore, we interpreted the mean decrease in accuracy as the variable (floral trait) importance, with a higher decrease meaning the model got worse when omitting a specific trait and thus this trait is more important in shaping the plant-pollinator interactions compared to traits with a lower decrease in accuracy.

We performed a single RF model encompassing all four elevations and seasons to view general patterns in floral trait importance. Additionally, RF's were done for each season and elevation separately to explore differences in trait importance along season and elevation. We ran 100 permutations of RF, comprising 200 to 500 trees each (decision made during plotting of the model) and two variables tested at each split (mtry) based on training of the model using the "train" function in the caret package (Kuhn, 2008). For each elevation and season the most important trait per primary pollinator group for extracted from these eight analyses. Per trait we listed the most common trait characteristic found in our dataset for the separate pollinator groups. Additionally, to evaluate the prediction accuracy of floral traits and trait combinations we used a full mountain (both seasons and elevations combined) RF model to predict the primary and secondary pollinators of plant species in each season and elevation based on the trait combinations. After which we were able to compare the predicted pollinator based on floral traits with the true primary or secondary pollinator. For the analyses we used the RANDOMFOREST package (Liaw & Wiener, 2002) in R 3.6.1 (R Core Team, 2019).

We used canonical correspondence analysis (CCA), a multivariate method to study the relationships between biological assemblages of species and their environment, in this case floral traits (ter Braak & Verdonschot, 1995). Using the Canoco 5 software (ter Braak & Šmilauer, 2012), we analysed patterns in spatiotemporal floral trait distribution and variance explained by the five most important traits found during the RF analyses. This allows us to further find differences in floral trait distribution and their relative importance between seasons and with elevational changes. We included all visitors touching the reproductive organs in these analyses, not only primary and secondary pollinator. We performed a single CCA to distinguish patterns between seasons and with elevation. Additionally, we performed eight separate forward selection CCA's (all four elevations and both seasons separately) to find the five most important trait characteristics for pollinators in each elevation and season, together with the variation explained by them.

Results

Based on 24h video recordings of flowers of 121 plant species with a complete set of floral traits, we identified 13,024 individual interactions of our selected potential pollinator groups, based on their contact with reproductive organs (See Materials and Methods, and Table **S2**). Using an extensive floral trait database (Table **S1**) for all co-occurring flowering species at our focal sites, we were able to identify the importance of each trait in predicting pollinator groups across elevation and seasons. Using Random Forest models (RF), we identified the five most important floral traits for shaping plant-pollinator interactions on Mount Cameroon based on their mean decrease in model accuracy: Shape, Sugar per flower, Colour, Size and Tube Length (Table **1**). Nevertheless, we found seasonal and elevational shifts in trait importance for specific functional groups of primary pollinators when using individual RF analyses per season and elevation (Table **2**).

Table 1 | Ten floral traits used in the Random Forest analyses ranked by their importance (mean decrease in accuracy and mean decrease in Gini index) in distinguishing the eleven potential primary pollinator groups averaged for the 100 RFs with 500 trees each. Note that some pollinator groups were not found to be primary pollinators of any plant species in any season or elevation and were therefore excluded.

	Mean Decrease Accuracy	Mean Decrease Gini	Beetle	Hoverfly	Other Fly	Carpenter bee	Honeybee	Wasp	Other Bee	Butterfly	Other Moth	Bird
Shape	0.019926	14.7037	0.026667	0.01554	0.035033	-0.007	0.005533	-0.01	0.021969	0.030667	0.042402	0.0181
Sugar per Flower	0.012072	20.26525	0.033333	0.030363	0.044145	0.018	0.015033	-0.007	-0.00039	0.014567	-0.03643	0.064033
Colour	0.011802	11.70518	-0.051	0.00632	0.056167	0.004	-0.01006	0.005	0.022869	0.010067	0.01776	-0.00847
Size	0.011793	18.72872	0.008333	0.01172	0.00766	0.007	-0.00178	0.003	0.020133	0.0426	0.014155	-0.00703
Tube Length	0.009278	13.0118	0.043333	0.005265	0.036919	0.015	-0.00933	-0.001	0.006333	0.011933	0.030868	-0.0195
Odour Strength	0.008588	3.617446	0.001333	-0.00491	-0.00216	0.019	0.019548	0.002	0.018386	-0.00657	0.02656	-0.00623
Anther Position	0.003122	3.248954	0.005	0.011909	0.003486	0	0.001454	0.003	0.000302	-0.00567	0.009452	-0.0109
Flower Position	0.001466	7.867185	0.005333	-0.00072	-0.0065	-0.012	-0.01507	-0.008	0.000483	-0.0056	0.042176	0.013367
Symmetry	0.001122	2.170926	0.013333	0.004342	0.000526	-0.002	0.003297	-0.011	-0.00207	-0.00457	0.005718	-0.009
Nectar Guides	-0.00074	2.625837	0.035	-0.00428	-0.00283	-0.006	0.000436	0	0.001247	0.000167	-0.00409	-0.01007

Table 2 | Most important trait per primary pollinator group according to the Random Forest analyses per season and elevation. The specific trait characteristics are given below each trait and are based on the highest occurrence of said traits in our dataset, for each specific season and elevation. For the continuous traits (size, tube length, or sugar per flower), the table shows whether the trait value is small, medium/moderate, or large/abundant compared to the rest of the database. Three groups were excluded from the table since they only were significant in a single season at a single elevation: Beetles (1,100m Dry: Shape *Open*), Carpenter bees (1,100m dry: Odour strength *Strong*) and Wasps (1,100m wet: Tube length *Small*).

	Hoverflies	Other Flies	Honeybees	Other Bees	Butterflies	Other Moths	Birds
2,200 DRY	Anther Position *Exposed/ Partially*	Sugar per flower *Small*	Anther position *Exposed*				
2,200 WET	Shape *Open/Stellate*	Shape *Open*				Colour *Green*	Sugar per flower *Moderate*
1,450 DRY	Colour *White*	Tube length *Medium*	Shape *Labiate/Trumpet/ Salverform/ Tube*	Tube length *Small*		Anther Position *Exposed*	Size *Large*
1,450 WET	Odour Strength *Weak-No*	Size *Small*		Colour *Orange*		Shape *Salverform*	Sugar per flower *Abundant*
1,100 DRY	Shape *Dish*	Symmetry *Actinomorphic*	Shape *Salverform*	Size *Medium*	Anther Position *Exposed*	Size *Medium*	Size *Large*
1,100 WET	Sugar per flower *Small*	Odour Strength *Strong*		Shape *Gullet*			Sugar per flower *Moderate*
650 DRY				Shape *Open*	Size *Large*	Shape *Dish/Salverform*	
650 WET				Shape *Open*			

These traits were also shown to be of similar importance when considering secondary pollinators (Table **S3**). These machine learning algorithms can also be used for predicting pollinators based on floral trait combinations. For both primary and secondary pollinators we found a high prediction success when comparing individual pollinator groups (Table **3**) and, along season and elevation (Table **4**). Nevertheless, secondary pollinators (i.e. the second most frequent visitor touching reproductive organs) in general were less well predicted (Table **3,4**).

Table 3 | Predictions of potential pollinators per potential pollinator group by comparing the trained Random Forest model based on the floral traits with the actual primary or secondary pollinator found visiting flowers of particular plant species. N/A means the pollinator group did not occur as primary or secondary pollinator of any plant species in the given elevation and season.

Order	Functional group	Primary Pollinator		Secondary Pollinator	
		# Plants[1]	% Correct[2]	# Plants[1]	% Correct[2]
Blattodea	Cockroaches	0	N/A	4	0.00
Chiroptera	Bats	0	N/A	1	0.00
Coleoptera	Beetles	5	80.00	20	60.00
Diptera	Hoverflies	49	69.39	24	79.17
Diptera	Other flies	22	63.64	23	82.61
Hymenoptera	Carpenter bees	6	66.67	3	33.33
Hymenoptera	Honeybees	17	88.24	7	42.86
Hymenoptera	Wasps	4	75.00	9	55.56
Hymenoptera	Other bees	58	84.48	30	60.00
Lepidoptera	Butterflies	12	91.67	16	43.75
Lepidoptera	Hawkmoths	3	66.67	6	100.00
Lepidoptera	Other Moths	21	85.71	13	53.85
Passeriformes	Birds	11	72.73	5	60.00
	Non-flying mammals	0	N/A	2	0.00
	Total	**208**	**78.37**	**163**	**61.35**

[1]Number of analysed plant species. [2]Percentage of correct pollinating group predictions.

Table 4 | Predictions of potential pollinator groups per season and elevation by comparing the trained Random Forest model with the actual primary or secondary pollinator found visiting flowers of particular plant species.

Elevation	Season	Primary Pollinator		Secondary Pollinator	
		# Plants[1]	% Correct[2]	# Plants[1]	% Correct[2]
2,200m	DRY	18	94.44	15	86.67
	WET	19	84.21	12	75.00
1,450m	DRY	24	70.83	21	42.86
	WET	26	80.77	21	71.43
1,100m	DRY	39	69.23	32	59.38
	WET	24	70.83	21	57.14
650m	DRY	37	86.49	26	53.85
	WET	21	71.43	15	60.00
Total		**208**	**77.88**	**163**	**61.35**

[1]Number of analysed plant species. [2]Percentage of correct pollinating group predictions.

Following the identification of the most important traits we used canonical correspondence analysis (CCA) to unveil significant shifts in the trait occurrence between dry and wet season and among particular elevations (Fig. **2a;** F=1.2, P=0.044). The CCA shows that closed (e.g. gullet) are more common in the wet season, whilst the distribution of nectar sugar per flower seems closely related to highest elevation (Fig. **2a**). To test trait importance at a finer scale; per season and elevation, we performed eight separate forward selection CCA's, with the five most important trait characteristics (Fig. **2b**; for significance see Table **S4**). The models revealed an increase in the explained variability towards higher elevations and from dry to wet season, with the exception of the wet season at 2,200m, thus showing the importance of floral traits in distinguishing potential pollinators in harsher conditions. Additionally, we found that during the dry season, tube length and floral size were of greater importance for attracting pollinators, while in the wet season, red and orange flowers were most attractive (Fig. **2b**). We also detected changes in trait importance with elevation, but these patterns were less apparent (Fig. **2b**). Additionally, the variation explained by individual floral traits was greatest towards the highest elevations, and in the wet season generally (except for the highest elevation). Nevertheless, it must be noted that no statistically significant traits were found in the wet season at the two lowest elevations.

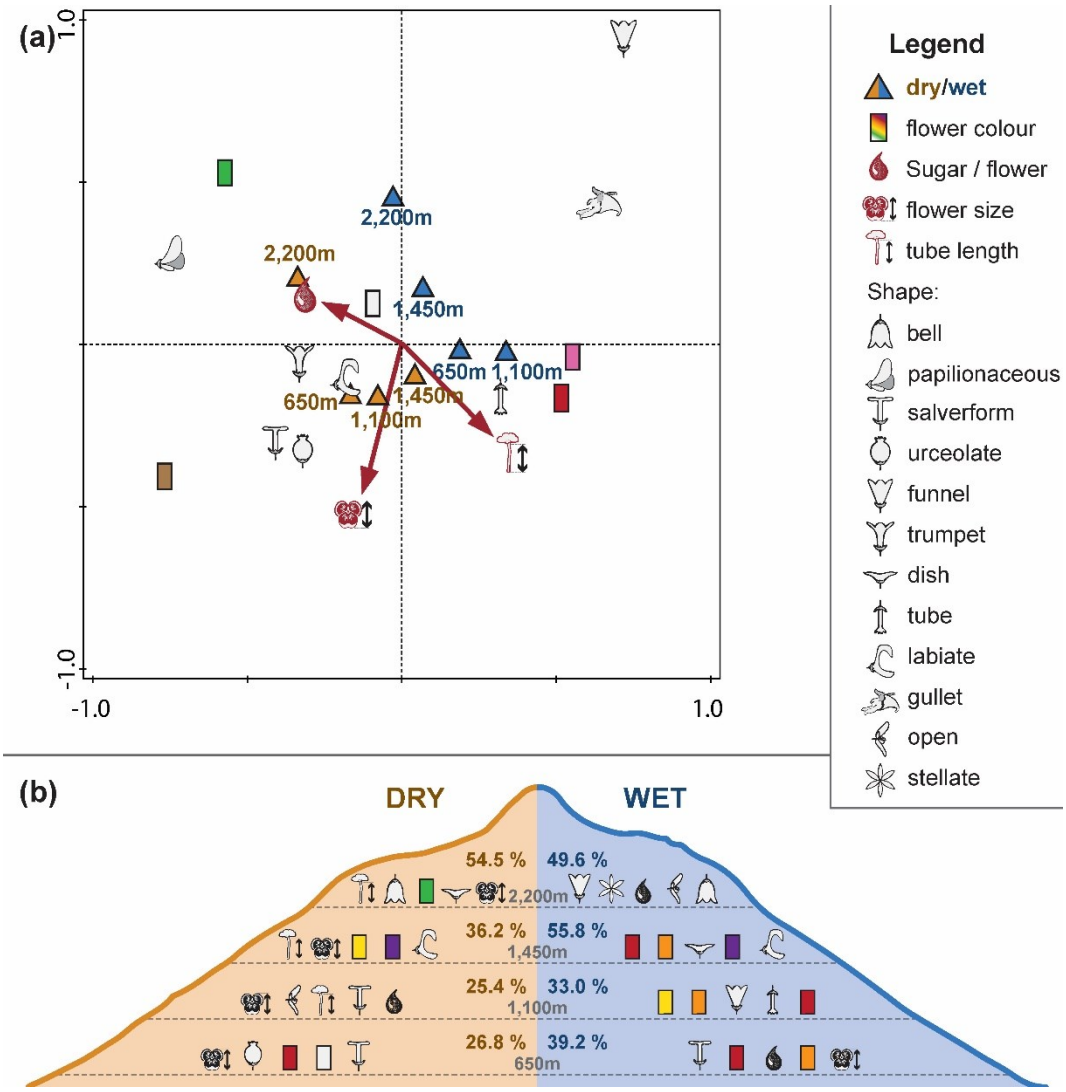

Fig. 2. a) CCA ordination diagram visualising the distribution of individual floral traits between season and along elevation (listed here as m a.s.l.). b) Summarised outcomes of individual CCA's of floral trait importance per elevation and season. Visualising the five floral trait characteristics with the highest ability to predict primary pollinators. The traits are sorted from left to right according to decreasing importance. Percentages in the middle states the variation explained by each model.

Discussion

We demonstrated that the relative importance of floral traits differs among the individual pollinator groups, and that there is spatiotemporal variability in floral trait distribution and importance (i.e. ability to predict pollinators) in the rainforest of Mount Cameroon.

The role of specific floral traits for flower visitors has been widely studied in numerous pollinator groups (e.g. Stang *et al.* 2009; Junker *et al.* 2013), with for example flower form and symmetry being important traits for flies (Lázaro, Hegland, & Totland, 2008). Compared to bees, flies are more common pollinators in the harsher conditions of higher elevations (Willmer, 2011). Traits related to this pollinator group (e.g. open/dish flowers) were found to be consistently important at the highest studied elevation, especially in the wet season (Fig. **2**, Table **2**). For bees, floral shape had the most predictive power. Open flowers were preferred in the lowest elevation (independent of the season), which might be explained by the flower offering a landing platform for bees, or by having exposed anthers, thus allowing for easy pollen collection. The huge variety in bee species, morphology and reward preferences (e.g. pollen, nectar and resin) allows them to exploit a wide range of floral designs (Willmer, 2011; Ollerton, 2017). This can also be seen in our study where flower shapes with a floral tube (salverform, tubular, trumpet) were the most important for honeybees at middle elevations (1,100m and 1,450m; Table **2**). In a global analysis of pollination syndromes (Ollerton *et al.*, 2009), bee and fly pollinated plants were predicted more accurately compared to other syndromes, while other studies did not show such patterns (Johnson, 2013). However, focusing only on the single most frequent visitor may not accurately depict true pollination, since visitation might be clouded by generalist visitors (Ollerton *et al.*, 2009; Junker & Blüthgen, 2010; Padyšáková *et al.*, 2013; Rosas-Guerrero *et al.*, 2014; Dellinger, 2020). Additionally, plants with morphologically generalised flowers are prone to this mismatch (Bartoš *et al.*, 2015).

We found the occurrence of rather closed flowers plays a bigger role at higher elevations, unlike Jacquemyn *et al.* (2005), who showed a sharp decrease of species displaying floral traits related to hawkmoth (long spur) or fly pollination (small or no spur) in orchid species along the elevational range of Réunion Island. We found the opposite pattern for floral traits considered important for fly pollination, since open/dish and dull looking (green/white colours) flowers were among the most important traits found at higher elevations (Fig. **2a,b**, Table **2**). These dull floral colours are often associated with hawkmoths (Faegri & van der Pijl, 1979; Willmer, 2011). However, tube length and scent have been shown to be more important traits for attracting hawkmoths (Willmer, 2011). We found shifts in the importance of corolla tube length only between dry and wet season and not along elevation (Fig. **2b**).

Seasonality also had a strong effect on floral trait occurrence and their ability to predict individual pollinator groups. The higher occurrence of closed flowers (gullet, funnel and tube) in the wet season might be explained by their role as shelter for visitors, limiting the dilution of nectar (Dafni, 1996; Aizen, 2003; Pacini & Nepi, 2007) or washing and/or damaging of pollen (Pacini & Franchi, 1984; Huang, Takahashi, & Dafni, 2002). Additionally, rainfall affects the flight ability of potential pollinators through increased thermoregulatory costs, rain avoidance or as environmental noise (Lawson & Rands, 2019). For birds and bats it has been shown that rainfall increases the energy cost of flight (Aizen, 2003; Voigt *et al.*, 2011; Ortega-Jimenez & Dudley, 2012). This effect is even greater for smaller insect visitors whose activity can be partially reduced or even completely impeded, by directly damaging them and reducing their abundance (Struck, 1994; Kishimoto-Yamada & Itioka, 2015; Maicher *et al.*, 2018, 2020; Chen *et al.*, 2019). Similarly, the higher energetic requirements in upper elevations resulting from lower temperatures, are consistent with the increased nectar production per flower with elevation. Nectar production was a consistently good predictor of pollinators in both the highest

(2,200 m) and lowest (650m) elevations during the wet season. In agreement with Aizen (2003) and Voigt *et al.* (2011), nectar sugar production was found to be especially important during the wet season for the most energy demanding pollinator group, birds. Since this group is more capable of dealing with harsher conditions (Cruden, 1972), floral traits associated with birds are expected to stand out. According to the individual RF analyses (Table **2**) nectar sugar per flower and size were more important for bird pollination. However, red flower colour, which is generally strongly associated with bird pollination (Chittka & Waser, 1997; Rodríguez-Gironés & Santamaría, 2004; Wester *et al.*, 2020), was found to be the third most important explanatory trait for birds in our study (Table **1**) and particularly important in the wet season (Fig. **2b**).

Furthermore, we also show spatiotemporal differences in the general ability of traits to predict pollinators. We found an increase in explained variation towards the higher elevations, with the notable exception of the wet season at 2200m. For now, we can only speculate that this might be due to higher pollen limitation in upper elevations (and thus stronger selection pressures; Ashman *et al.* 2004; Knight *et al.* 2005) or that the plant and pollinator interactions, abundance and richness effects can be of influence.

Floral traits were better predictors of pollinators in the wet season, with the exception of the highest elevation. Souza *et al.* (2018) suggested that differences in resource availability in individual seasons can result in differences in interspecific competition among pollinators and niche overlaps. This suggestion was based on their observations from grassland and shrubby vegetation in the Pantanal and Cerrado ecosystems in Central Brazil, where the dry season was associated with a lack of resources and thus potentially higher levels of competition. In contrast, we found nectar production per hectare on Mount Cameroon was several times lower in the wet season (unpublished results). Nevertheless, we assume that, similar to Souza *et al.* (2018), higher competition and reduced niche overlap in the period of resource shortage can play a role,

and these processes can result in higher plant specialisation and therefore greater predictability of pollinators by floral traits.

As we have shown, not all floral traits are equally important for individual pollinator groups and even traits that were not associated with traditional syndromes were found to be potentially important for predicting pollinators (Dellinger, 2020). From a methodological standpoint, machine learning approaches (such as RF) offer an avenue for dealing with such shifts in individual trait importance, increasing our predictive power. As a result, we have begun to see its application in several studies on trait selection, matching and syndrome testing (Johnson, 2013; Dellinger *et al.*, 2019a; Pichler *et al.*, 2020). However, the necessity to remove incomplete and collinear traits when applying this trait based method has also been apparent (Johnson, 2013; Dellinger *et al.*, 2019a; Pichler *et al.*, 2020). Regardless, even without a priori floral trait selection, the robustness of RF seems to allow for an ecologically realistic inference of pollinator predictability (Pichler *et al.*, 2020).

Conclusion

Our results showed the importance of floral traits in shaping plant-pollinator interactions in the understudied Afrotropics, with traits being more important towards the harsher conditions of higher elevations and wet seasons. These shifts in floral trait dependence between season and elevation show the importance of including spatiotemporal factors within pollination studies. The pollination syndromes will, in turn, vary in their ability to predict primary pollinators for different conditions and for the different pollinating groups. Additionally, shifts in the importance of specific discriminative traits for individual pollinator groups, together with spatiotemporal differences within these groups, suggests that following a complex predetermined list of equally important traits can be problematic for classifying potential pollinators. Using Random Forest models to categorise plants by floral traits and predict

potential interactions enabled us to abandon the traditional pollination syndrome approach. Although the concept of pollination syndromes is still highly interesting, we propose stepping back from the pollination syndromes into individual traits and first improve our trait-based understanding of plant-pollinator interactions at the community level under different spatiotemporal and environmental conditions (Schmid *et al.*, 2015).

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Supplementary information

Supplementary Table 1. Floral traits measured in the field for analyses with explanations of the categories.

Floral trait	Categories	Note	Included in analyses
Shape	Bell, Bowl, Dish, Funnel, Gullet, Labiate, Open, Papilionaceous, Salverform, Stellate, Trumpet, Tube, Urceolate	As defined by Faegri and van der Pijl (1979), Ramirez 2003 and Ollerton and Watts 2002)	Yes
Symmetry	Actinomorphic / zygomorphic		Yes
Size (cm)	Quantitative data	Zygomorphic flowers were measured along the horizontal and vertical axis	Yes
Flower position	Horizontal, Pendant, Upright, All	All is a separate group with flowers of a plant growing in all three directions	Yes
Anther position	Exposed, partially exposed, Hidden		Yes
Tube width (cm)	Quantitative continuous data	Measured at the middle of the tube. In some cases, it was measured at the opening of the tube	No, correlated to Tube length (Spearman's correlation: 0.8)
Tube length (cm)	Quantitative continuous data		Yes
Odour strength	Strong, Weak-No	Measured by observer smelling, therefore categories were kept simple.	Yes
Colour	Blue, Brown, Green, Orange, Pink, Purple, Red, White, Yellow	Based on visual inspection of flower	Yes
Nectar guides	Presence/Absence	Based on visual inspection of flower	Yes
Sugar amount per flower (mg)	Quantitative continuous data	Adjusted amount of sugar per sample to amount per flower based on HPLC or direct measurement using a pocket refractometer	Yes

Supplementary Table 2. Summary of the number of plant species recorded per season and elevation including total hours of recordings and number of visits per pollinator group. Note that in the Random Forest analyses only primary and secondary pollinators were used (i.e. first and second most frequent visitor touching plant reproductive organs), therefore some groups, although present here, might be missing from the RF analyses.

Elevation	Season	Plants	Recording hours	Cockroaches	Bats	Beetles	Hoverflies	Other flies	Carpenter bees	Honeybees	Wasps	Other bees	Butterflies	Hawkmoths	Other moths	Birds	Non-flying mammals
2,200m	DRY	18	1910			37	219	136		1696	4	3	4	23	78	5	5
	WET	19	1385			3	112	173		46	1	2		4	23	43	
1,450m	DRY	24	2139	6	39	68	183	130	31	489	6	141	161	27	206	18	3
	WET	26	1745			5	836	196		5		218	25	3	122	98	1
1,100m	DRY	39	2912	17		52	181	100	71	85	60	314	137	12	163	8	
	WET	24	1708			9	323	211	6		41	131	43	6	33	51	1
650m	DRY	37	2651	2		29	19	12	9	117	33	1084	113	12	152	4	
	WET	21	1636	1		14	12	251		1	136	1001	65	10	27		

Supplementary Table 3. Ten floral traits used in the Random Forest analyses ranked by their importance (mean decrease in accuracy and mean decrease in Gini index) in distinguishing the eleven potential secondary pollinator groups averaged for the 100 RFs with 500 trees each. Note that some pollinator groups were not found to be secondary pollinators of any plant species and were therefore excluded from the analysis.

	Mean Decrease Accuracy	Mean Decrease Gini	Beetle	Hoverfly	Other Fly	Carpenter bee	Honeybee	Wasp	Other Bee	Butterfly	Hawkmoth	Other Moth	Bird
Size	2.72	17.05	2.35	2.59	2.26	2.35	-2.44	-1.82	0.83	2	-0.26	-3.12	-1.16
Sugar per flower	-0.32	16.55	-0.56	0.35	0.82	0	-2.28	-1.49	-0.08	1.53	1.25	-1.82	-3.28
Shape	4.66	13.19	0.36	2.11	3.61	-1.67	-1	-0.06	-0.46	3.59	1.78	-0.95	1.34
Tube Length	1.67	12.94	1.86	-3.55	3.37	2.01	-1	-3.05	0.21	1.1	5.88	0.66	-1.64
Colour	-3.02	9.71	0.83	0.46	-3.53	-3.35	-0.45	-1.94	-2.02	-1.43	3.62	-0.03	-1.42
Flower Position	-2.17	6.46	-4.61	-3.8	0.64	3.19	1	-1.26	2.43	0.74	-0.95	-2	1
Odour Strength	2.01	3.98	-2.55	-0.44	5.56	-2.25	0	-0.56	-0.71	2.25	2.82	-0.44	-3.21
Anther Position	0.17	3.2	1.29	1.48	1.16	0	0	-1.64	-2.2	-0.58	-2.59	-0.15	0
Symmetry	0.56	2.42	0.1	0.69	-1.71	1.74	0	0.93	2.37	2.75	-1.87	-0.46	1
Nectar Guides	2.13	2.39	1.2	-0.59	0.79	1	0	1	3.73	-0.54	-1.48	1.88	0

Supplementary Table 4. Significance values of individual canonical correspondence Analyses (CCA) per season and elevation. This table supplements Fig. 2B, it shows the significance of the ranked traits per season and elevation. Rank (1-5), explained variation (%), Pseudo-F and P value are displayed.

			Shape									**Colour**						**Size**	**Tube length**	**Sugar per flower**
			Bell	*Salverform*	*Urceolate*	*Funnel*	*Dish*	*Tube*	*Labiate*	*Open*	*Stellate*	*Green*	*Orange*	*Purple*	*Red*	*White*	*Yellow*			
2,200 m	**Dry**	Rank	2				4					3						5	1	
		Explained variation	11				14.5					9.7						6.5	12.9	
		F	2.2				3.6					2						1.7	2.4	
		P	0.128				**0.008**					0.144						0.186	0.078	
	Wet	Rank	5			1				4	2									3
		Explained variation	5.5			16.1				5.2	12.6									10.2
		F	1.4			3.3				1.3	2.8									2.5
		P	0.22			0.07				0.296	**0.048**									0.07
1,450 m	**Dry**	Rank							5					4			3	2	1	
		Explained variation							6.4					6			7.4	7.6	8.9	
		F							1.9					1.7			2	2	2.2	
		P							**0.036**					**0.048**			**0.04**	**0.044**	**0.004**	
	Wet	Rank					3		5				2	4	1					
		Explained variation					9.9		13.6				11.8	6.6	13.9					
		F					3.5		6.4				3.8	2.5	4					
		P					0.072		**0.002**				**0.028**	0.062	**0.006**					
1,100 m	**Dry**	Rank		4						2								1	3	5
		Explained variation		4.3						6.5								5.8	4.2	4.6
		F		1.9						2.7								2.3	1.8	2.1
		P		**0.044**						**0.002**								**0.012**	**0.042**	0.068
	Wet	Rank				3		4					2		5		1			
		Explained variation				6		5.5					7.3		5.56		8.5			
		F				1.5		1.4					1.8		1.5		2.1			
		P				0.204		0.17					0.072		0.118		0.078			
650 m	**Dry**	Rank		5	2										3	4		1		
		Explained variation		5.1	5.8										5.4	3.7		6.8		
		F		2.3	2.4										2.3	1.6		2.7		
		P		**0.02**	0.108										**0.028**	0.064		**0.016**		
	Wet	Rank		1									4		2			5		3
		Explained variation		13.2									5.8		7.5			6.1		6.6
		F		2.9									1.4		1.7			1.5		1.5
		P		0.056									0.164		0.168			0.17		0.138

CHAPTER II

Mertens JEJ, Brisson L, Janeček Š, **Klomberg Y**, Maicher V, Sáfián S, Potocký P, Delabye S, Kobe IN, Pyrcz T, Tropek R (Manuscript). Elevational and seasonal patterns of butterflies and hawkmoths in plant-pollinator networks in tropical rainforests of Mount Cameroon.

YK was strongly involved in the fieldwork and processing and management of the data. Additionally, YK was in charge of measuring floral traits, managing the trait datasets and contributed to writing of the manuscript.

Elevational and seasonal patterns of butterflies and hawkmoths in plant-pollinator networks in tropical rainforests of Mount Cameroon

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Abstract

Butterflies and moths are well-visible flower visitors. Nevertheless, almost no quantification of their role in plant-pollinator interactions exists at a community level, especially from tropical rainforests. Moreover, we have virtually no knowledge on environmental and other factors affecting lepidopteran flower visits.

We focused on the role of butterflies and hawkmoths as flower visitors in tropical rainforests of Mount Cameroon, especially on its elevational and seasonal changes. We also analysed their preferences to selected floral traits, with a specific focus on pollination syndromes. We video-recorded flower visitors of 1,115 specimens of 212 plant species (>26,000 recording hrs) along the complete elevational gradient of rainforests in two main seasons, and compared frequencies of flower-visiting lepidopterans to other visitors. We compared characteristics of plant-lepidopteran networks among elevations and seasons, and analysed patterns of selected lepidopteran traits. Finally, we analysed inter-family differences in their floral preferences.

Altogether, we recorded 734 flower visits by 80 butterflies and 27 hawkmoth species, representing only ~4% of all 18,439 flower visits. Although lepidopterans visited only a third species, they appeared key visitors of several plants. The most flower visits by lepidopterans were recorded in mid-elevations and dry season, mirroring the general patterns of lepidopteran diversity. The networks showed no apparent elevational or seasonal patterns, probably because of the surprisingly high specialisation of interactions in all networks. Significant non-linear changes of proboscis and forewing lengths were found along elevation, and long-proboscid hesperiid butterflies visited flowers with longer tubes or spurs. Substantial differences in floral preferences were found between sphingids, and papilionid, nymphalid and lycaenid butterflies, revealing importance of nectar production, floral size and shape for sphingids, and floral colour for butterflies.

Butterflies and hawkmoths were confirmed as relatively minor visitors of tropical forest flowers, although they seemed crucial for pollination of some plant species. Moreover, the revealed floral preferences and trait-matchings confirmed a potential of some lepidopteran families to drive floral evolution in tropical ecosystems.

Introduction

Recently, pollination research shifted from detailed studies of single pollination systems to network approaches. Nevertheless, most complex studies of individual pollinator groups' role in plant-pollinator networks have focused on bees or hoverflies (Klecka *et al.*, 2018; Classen *et al.*, 2020), whilst the other flower visitors have often been excluded or side-lined. Although some less abundant groups play important roles in pollination systems, as secondary pollinators, nectar thieves and competitors, or even as key pollinators of specialised plants (Wardhaugh, 2015; Hahn & Brühl, 2016; Ollerton, 2017; Martínez-Adriano, Díaz-Castelazo, & Aguirre-Jaimes, 2018; Mertens *et al.*, 2020), their importance in plant-pollinator networks remains understudied, especially in tropical forests.

Compared to bees and flies, butterflies and hawkmoths represent minor pollinators in probably all terrestrial ecosystems (Wardhaugh, 2015; Ollerton, 2017). Both groups are often regarded as generalised nectar feeders visiting all available nectar-rich flowers (Willmer, 2011; Johnson *et al.*, 2017). Even hawkmoths, considered as efficient pollinators strongly affecting floral evolution already since Darwin (1862), were recently revealed as opportunistic nectar thieves of many flowers (e.g. Martins & Johnson, 2013; Fox *et al.*, 2015). However, some butterflies(e.g. Arroyo *et al.*, 2007; Santos *et al.*, 2020; Mertens *et al.*, 2020) and moths (e.g. Fleming & Holland, 1998; Hahn & Brühl, 2016; Johnson *et al.*, 2017; Skogen *et al.*, 2019) are key pollinators of specialised plants.

Individual lepidopteran groups differ in their morphological and behavioural adaptations to pollination. Among butterflies, papilionids, pierids, and some groups of nymphalids and hesperiids use their long proboscis to feed on nectar from deep flowers, whilst many lycaenids, riodinids, and some smaller clades within the mentioned families bear small proboscis unable to reach nectar in specialised flowers (Corbet, 2000; Tiple, Khurad, & Dennis, 2009). In moths, besides highly specialised long-proboscid groups, such as most sphingids and noctuids, adults of many groups have dysfunctional or even no proboscis (Willmer, 2011). Such differences hamper any attempts at quantifying the general pollination role of lepidopterans. Especially at the community level, the relative importance of butterflies and hawkmoths as pollinators is understudied.

Plants also differ in their adaptation to butterfly or moth pollination. The pollination syndrome hypothesis (Faegri & van der Pijl, 1979) expects some plants to evolve certain traits to attract the two groups. Psychophily hypothesises the adaptation for butterfly-pollination, whilst sphingophily defines hawkmoth-pollinated flowers and is distinguished from phalaenophily, i.e. pollination by any other moths (Faegri & van der Pijl, 1979; Willmer, 2011). Consistently, butterflies and hawkmoths should prefer large conspicuous flowers or inflorescences (Arroyo *et al.*, 2007; Mitchell, Dötterl, & Schaefer, 2015). Nocturnal hawkmoths rely equally on colour and scent when foraging, often preferring light colours (such as white or cream) better distinguishable in dark, and strong sweet scents (Kelber, Balkenius, & Warrant, 2003; Glover, 2011). This is in contrast with butterflies typically preferring bright flower colours, such as red or orange, above scent (Ômura & Honda, 2005), although sweet and fruity scents were also included into the psychophily (Willmer, 2011). Nevertheless, the colour preference strongly varies among butterfly families and species (Yurtsever, Okyar, & Guler, 2010; Pohl, Van Wyk, & Campbell, 2011). Their size and proboscis length also influence flower preferences (Tiple *et al.*, 2009). Small short-proboscid lycaenids avoid long-tubed flowers but

can visit small solitary flowers, long-proboscid papilionids or pierids, often larger and more energy-demanding, prefer massed nectar-rich flowers (Corbet, 2000; Tiple *et al.*, 2009). Long-proboscid hawkmoths can visit both long and short tubed flowers (Johnson *et al.*, 2017).

Elevation and seasonality, representing various environmental and ecological gradients, influence patterns in biotic interactions (Poisot, Stouffer, & Gravel, 2015; Klomberg *et al.*, 2020). The role, relative proportions in communities, and specific adaptations of pollinator groups shift under differing environmental conditions, such as temperature, solar radiation, and precipitation (e.g. Ollerton et al., 2006; Klomberg et al., 2020). Unfortunately, neither elevational nor seasonal patterns of tropical lepidopteran role in pollination networks were studied, except a few case studies of individual plant species (e.g. Mertens et al., 2020). However, we can expect some correlations of their role in networks with their general diversity patterns. We have no community-wide studies on characteristics of these lepidopteran-plant pollination networks in any tropical area.

Our study focuses on flower-visiting butterflies and hawkmoths, the two relatively minor groups of pollinators often overlooked in network studies, yet easily identifiable. The primary Afrotropical rainforests covering Mount Cameroon from nearly sea level to the natural timberline offer a unique elevational gradient, with distinct dry and wet seasons. Based on rich community-wide datasets sampled along the elevational gradient and during the two seasons, we set the following aims: (1) To evaluate the role of flower-visiting butterflies and hawkmoths in plant-pollinator networks and understand how elevation and seasonality affect their relative importance in pollination communities. (2) To analyse potential elevational and seasonal changes in structure of the pollination networks, with a specific focus on specialisation. (3) To assess the butterfly and hawkmoths preferences to floral traits, as well as to test potential trait-matching between flowers and their visitors. (4) To test potential relationship of proboscis length and specialisation of butterflies and moths in visited flowers. We hypothesise that

butterflies and hawkmoths represent only a small proportion of the flower-visiting communities, although we expect it will be higher in lowlands and in dry season where and when both groups are more abundant and diverse on Mount Cameroon (Maicher *et al.*, 2018, 2020). We also expect higher specialisation in communities with more flower-visiting lepidopteran species, such as in some other insect-plant interactions (e.g. Rasmann et al., 2018). We expect both groups to be important pollinators of some specialised plants. We hypothesise preferences to some traits previously included in the psychophilous and sphingophilous syndromes, although we expect some predicted traits to be less important. We also expect substantial differences in the mentioned aims and hypotheses among lepidopteran families.

Materials and Methods

Study area

Mount Cameroon (4,095 m a.s.l.) is an active volcano in the Southwest Region, Cameroon, West/Central Africa. Primary tropical rainforests cover its southwestern flanks, where the study was performed, from lowlands (above human encroachment at ca. 300 m a.s.l.) up to the natural timberline (ca. 2200 m a.s.l.). As the mountain is located within the 'Guinean forests of West Africa' biodiversity hotspot, it holds an extraordinarily high biodiversity of numerous taxa, including butterflies (Larsen, 2005), hawkmoths (Ballesteros-Mejia *et al.*, 2013), and plants (Cheek *et al.*, 1996). The mountain belongs among the wettest places in the world and experiences distinct dry (December–February) and wet seasons (June–September; Maicher et al., 2018, 2020)). The Atlantic Ocean-facing southwestern lowlands receive large amounts of rainfall (>12,000 mm annually), most of which during the wet season (>2,500 mm monthly), and rarely any rain during the dry season (Maicher et al. 2020). To characterise changes in plant-pollinator interactions along elevation and season, we studied four sites on the southwestern slope at 650, 1,100, 1,450 and 2,200 m a.s.l. The butterfly and hawkmoth species

richness data includes additional sampling sites at 30, 350 and 1,850 m a.s.l. (Table 1). For more details on the study sites, see Maicher et al. (2020).

Study groups and their biodiversity patterns

This study focused on butterflies (Lepidoptera: Papilionoidea) and hawkmoths (Lepidoptera: Sphingidae; hereafter referred to as sphingids). For part of the analyses, butterflies were split up in their families (Hesperiidae, Papilionidae, Pieridae, Lycaenidae, and Nymphalidae; hereafter referred to as hesperiids, papilionids, pierids, lycaenids, and nymphalids). All butterflies and sphingids were represented in the *flower visitation* and *floral preferences* parts of the study. However, the effects of elevation and season on visitor size and proboscis length were analysed for papilionids, hesperiids and sphingids only, because the other groups' traits were not measured in the field. We actively inventoried Lepidoptera along the complete elevation of Mt. Cameroon (i.e. at seven elevations from 30 to 2,200 m a.s.l.), using the checklist approach. For this purposes, we applied intensive hand-catching (our unpublished data) and bait-trapping (data from Maicher et al. 2020) of butterflies, whereas standardised light-attraction of sphingids (data from Maicher et al. 2020). These data were further supplemented by a few additional species found only in the video recordings described below.

Flower visitation

We recorded flower-visiting lepidopterans at the four elevations (650, 1,150, 1,450 and 2,250 m a.s.l.), along six transects (200×10 m) per elevation established to characterise the local vegetation heterogeneity (Klomberg *et al.*, 2020). Along those transects, we recorded flower visitors of all plant species flowering during our fieldwork (two weeks during dry and wet seasons at each elevation; Table 1) using security cameras with IR night-vision (Vivotek IB8367RT). We positioned the cameras 0.5–1.5 m from the flowers or inflorescences and

camouflaged their surfaces. We recorded flowering plants at all vegetation layers from understorey to canopy, using ladders and tree climbing to reach higher strata. Five individuals of each plant species were recorded, each for a 24hour session. The individual replicates were separated in space (different transects) and time (different days). During the first week, we added any plant species that have been or just started flowering. The second week served towards completing the necessary five replicates and no more species were added to the study. Whenever insufficient individuals flowered along the transects, we searched the adjoining area.

We observed all flower visitors from the video recordings either through semi-autonomic motion detection with Motion Meerkat 2.0.5 (Weinstein, 2015) when conditions allowed, or manually through sped-up playback. We identified all butterflies and sphingids to (morpho)species using various available literature and our reference collection. For each visiting Lepidoptera, we determined whether they touched the plant's reproductive organs (anthers, stigmata, or both) to distinguish potential pollinators from other visitors. The recorded interactions were used to reconstruct interaction networks among flowering plants and visiting lepidopterans (hereafter simplified to *plant-lepidopteran networks* or *networks*) for each elevation and season (i.e. eight networks). We used visitation frequency (i.e. number of interactions of each species per plant species during 24h) in each of the eight networks. This controls for differences in total recording time between plant species in the few cases we failed to find enough replicates or the recordings were shorter because of dying flowers or technical failures.

To visualise and characterise the eight plant-lepidopteran networks, we used the *bipartite* package (Dormann *et al.*, 2009) in R 3.5.3 (R Core Team, 2019). We quantified network *connectance* (Jordano, 1987), network-level *H_2' specialisation* (Blüthgen, Menzel, & Blüthgen, 2006), *Q modularity* (Dormann & Strauss, 2014), and *NODF nestedness* (Almeida-Neto *et al.*, 2008). We calculated each metric firstly including all floral visitors, and secondly

only with the subset of visitors touching the plant's reproductive organs. Because of the highly limited number of replicates (each combination of elevation and season was characterised by a single network), any possible elevational and seasonal patterns of the network characteristics were checked by a direct comparison of values, i.e. without any statistics. Finally, we calculated *d' specialisation* (Blüthgen *et al.*, 2006) of each lepidopteran species in each network. The relationship of the species-level specialisation of lepidopterans to elevation and season was analysed by a linear mixed-effect model (LMM) with specialisation of individual lepidopteran species in particular networks as a continuous response variable, and with elevation and season as categorical explanatory variables. Individual lepidopteran families were included as a categorical random-effect factor to correct for the inter-family variability.

Relationship between floral and lepidopteran traits

We measured six floral traits of 174 plant species included in the plant-pollinator networks: *symmetry* (actino- or zygomorphic), prevailing *floral colour*, *corolla width*, floral *tube length* (distance from the flower opening to its base, or tip of the spur when present), and *nectar sugar* (total mass of sugars produced by a flower during 24h; the sampling protocol followed Bartoš et al. 2020).

We measured eight morphometric traits of 1,665 specimens of 130 lepidopteran species (75 hesperiids, 15 papilionids, and 40 sphingids) collected during the project. Directly in the field, we *weighted* fresh specimens and cut their proboscides for later measurement. The collected specimens were mounted and photographed at the Nature Education Centre, Jagiellonian University, Krakow. On these photographs, we measured *forewing length* and *width*, *body length*, and *thorax width*, *lengths of fore-*, *mid-* and *hindleg*, and *proboscis* (Fig. S1) in ImageJ2 (Rueden *et al.*, 2017). We assessed the lepidopteran trait collinearity by multiple

regression and selected the proboscis and forewing lengths as the other traits' proxies (Table S2).

We analysed patterns of the proboscis and forewing lengths in communities of all lepidopterans measured at different elevations by LMM. The average trait values per species were used as response variable (log-transformed as the data showed a lognormal distribution), elevation as categorical fixed-effect variable, and lepidopteran families as random-effect variable to correct for inter-family variability. Consequently, we analysed elevational and seasonal (fixed-effect variables) differences in the proboscis or forewing lengths (response variables) in flower-visiting lepidopteran species only. This dataset involved 34 measured lepidopteran species recorded during flower visits (19 hesperiids, 7 papilionids, 8 sphingids). In both analyses, we applied AICc (AIC corrected for small samples, Hurvich and Tsai 1993) to select the most plausible models. Due to the high variability in sample sizes, no post-hoc tests were performed. Finally, we tested correlation between the proboscis and forewing length of lepidopterans and their d' specialisation using Spearman's rank coefficients.

Floral preferences

We assessed how the floral preferences to particular floral traits differ among the six focal lepidopteran families by ordination analyses in Canoco 5 (ter Braak & Šmilauer, 2012). All five measured flower traits served as explanatory variables towards the visitation frequencies by lepidopteran species (response variable). Based on the gradient lengths, two RDA models were selected and tested using 999 Monte Carlo permutations (Šmilauer & Lepš, 2014). Firstly, to assess lepidopteran preferences within the whole local community of flowering plants, we included all plant species for which we measured the traits (n=173). Subsequently, we analysed lepidopteran preferences only among the visited plant traits (n=63). Finally, we tested whether lepidopterans with longer proboscides visit flowers with longer corolla tubes by correlating the

average proboscis length of the 34 measured lepidopteran visitors with the corolla tube length of the visited plant species using Spearman's rank coefficient.

Results

Altogether, we recorded 431 butterfly and 40 sphingid species on Mount Cameroon (Table 1; Fig. 1a). Nymphalids comprised the most species across the gradient, followed by lycaenids in the lowest elevations and hesperiids at the mid-elevations (650 m to 1,450 m a.s.l.). The total species richness of lepidopterans, as well as of lycaenids, showed gradual decrease along elevation (Fig 1a), all other butterflies showed the low plateau pattern (*sensu* McCain and Grytnes 2010), whilst sphingids peaked at 350 m a.s.l. (Fig. 1a).

Altogether, we recorded 1,115 individuals of 212 flowering plant species for a combined 26,138 hours (~2.98 years) of video footage, during which we observed 734 individuals of 80 butterfly and 27 sphingid species visiting 71 plant species. These visits represented 4% of all 18,439 flower visits recorded on the observed plants. Bees, flies, beetles, and other moths were more common flower visitors than butterflies (Klomberg *et al.*, 2020). Wasps, nectarivorous birds and carpenter bees were more common visitors than sphingids, followed by cockroaches and mammals (Klomberg *et al.*, 2020). Still, butterflies and sphingids were among the two most common flower visitors for some plant species, such as *Scadoxus cinnabarinus* (Amaryllidaceae), *Distephanus biafrae* and *Melanthera scandens* (both Asteraceae), and *Cordia aurantiaca* (Boraginaceae) for butterflies; and *Anthocleista scandens* (Gentianaceae) for sphingids (Table S4). From these, 700 lepidopteran visitors touched the plant reproductive organs (see Table S3 for a taxonomic and spatiotemporal overview). Due to the small difference between 'pollinators' and 'all visitors', results of our analyses with both datasets were nearly identical, and we only report all visiting records, i.e. the visitors' point of view.

Table 1. Sites on Mount Cameroon sampled for butterflies and sphingids. 'n.a.' states for data not available for particular sites.

Site				Sampled period		Number of all species		Species in pollination networks (dry / wet seasons)			
Elevation (a.s.l.)	**Latitude**	**Longitude**	**Vegetation type**	**Checklist**	**Networks (dry/wet)**	**Butterflies**	**Sphingids**	**Butterflies**	**Sphingids**	**All plants**	**Visited plants**
30 m	N 03.9818°	E 09.2625°	Coastal forest	Dec 2014, Jan 2015, May 2015, Oct 2017	n.a.	282	5	n.a.	n.a.	n.a.	n.a.
350 m	N 04.0899°	E 09.0517°	Mosaic of primary and secondary lowland forest	Dec 2014, Apr 2015, Jan/Feb 2016	n.a.	189	28	n.a.	n.a.	n.a.	n.a.
650 m	N 04.1022°	E 09.0630°	Primary lowland forest	Nov/Dec 2014, Apr 2015, Jan/Feb 2016	Jan 2018 / Aug 2018	189	20	32 / 14	5 / 6	62 / 42	19 / 11
1,100 m	N 04.1175°	E 09.0709°	Upland forest disturbed by elephants	Dec 2014, Jan 2015, Apr 2015, Jan/Feb 2016	Feb 2018 / Sep 2018	161	8	38 / 7	7 / 4	61 / 32	25 / 12
1,450 m	N 04.1443°	E 09.0717°	Submontane forest disturbed by elephants	Nov 2016, Feb 2017, Apr/May 2017	Feb 2017 / Sep 2017	64	7	13 / 7	9 / 4	42 / 35	17 / 6
1,850 m	N 04.1453°	E 09.0870°	Montane forest disturbed by elephants	Nov 2016, Feb 2017, Apr 2017	n.a.	12	7	n.a.	n.a.	n.a.	n.a.
2,200 m	N 04.1428°	E 09.1225°	Montane forest close to timberline	Nov 2016, Jan/Feb 2017, Apr 2017	Jan 2017 / Aug 2017	13	3	3 / 0	2 / 1	22 / 28	6 / 2
					TOTAL	**431**	**40**	**80** (69 / 25)	**26** (19 / 12)	**212** (144 / 106)	**71** (54 / 26)

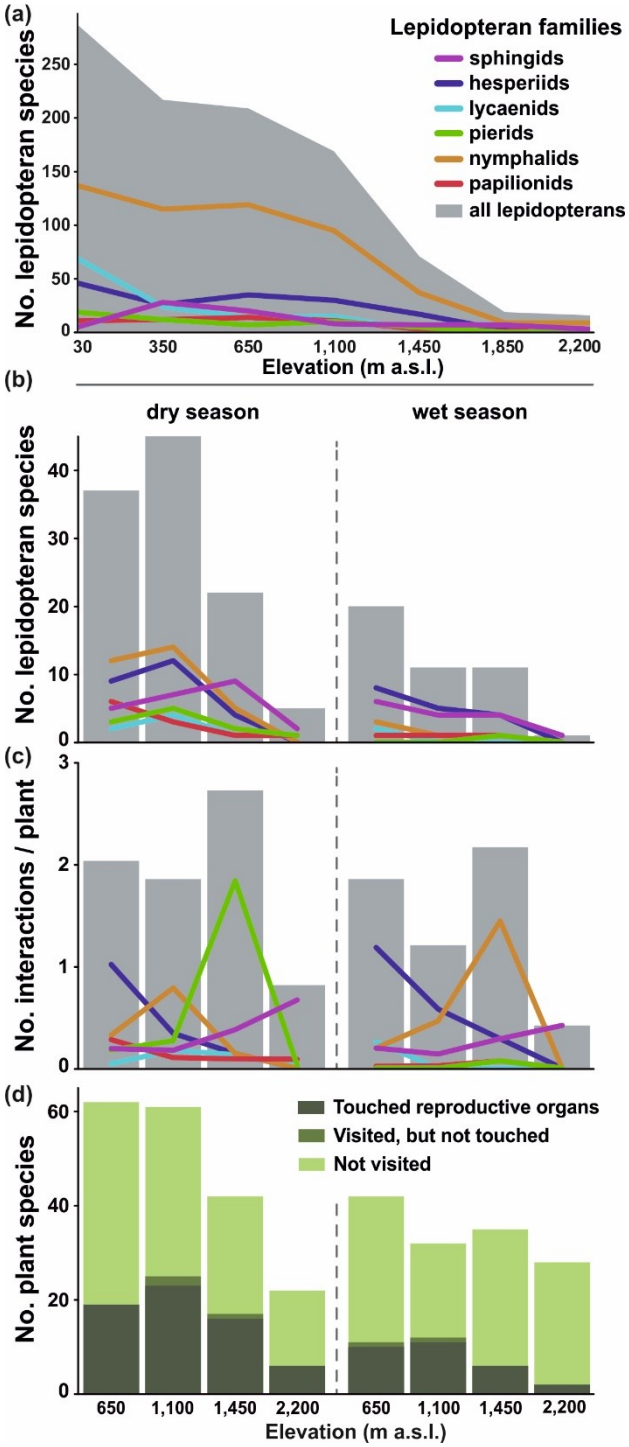

Figure 1. Overview of (a) lepidopteran species richness along the elevational gradient of Mount Cameroon, (b) total number of flower-visiting lepidopteran species at each elevation and season, (c) interaction frequency per plant and 24hrs, and (d) plant species where lepidopterans touched reproductive organs, where lepidopterans visited without touching any reproductive organs, and where lepidopteran visits were not recorded. Grey shading in (a-c) denotes the sum of all lepidopteran taxa, coloured lines represent particular families.

We recorded the highest species richness of both lepidopteran flower visitors and lepidopteran-visited plants at 1,100 m a.s.l. during dry season (Table 1; Fig. 1b). Species richness of visiting lepidopterans decreased towards the higher elevations and during wet season; at 2,200 m a.s.l. during wet season we recorded only a sphingid species visiting two plant species (Fig. 1b). In accordance, species richness of all flowering plants decreased towards the higher elevations and in wet season. Yet, the highest elevation was species-richer

during wet than during dry season. Overall, lepidopterans visited a lower proportion of flowering plant species during the wet season (wet season mean = 0.204 (±0.114) vs. dry season mean = 0.334 (±0.054); Fig. 1d).

The visitation frequency varied among the visitor families (Fig. 1c). Hesperiids were less frequent towards the higher elevations in both seasons. Papilionids followed such pattern in dry season but represented a generally small proportion of visitors in wet season. Lycaenids were generally uncommon flower visitors with only small spatiotemporal differences. Pierids expressed a peak in frequency at 1,450 m a.s.l. during dry season (driven by *Mylothris* cf. *hilara* frequently visiting *Distephanus biafrae*). Nymphalids expressed a similar peak at 1,450 m a.s.l. during wet season (driven by *Vanessula milca* visiting *Melanthera scandens*). Finally, sphingids visited flowers more frequently towards the higher elevations in both seasons (Fig. 1c).

We found no apparent general pattern in turnover of flower-visiting lepidopterans and lepidopteran-visited plants among the studied elevations and seasons (Fig. S2). The higher elevations shared less plant species with the lower elevations as well as between each other. The visitor community shared most species between 1,100 m and 1,450 m, followed by 1,450 m and 2,200 m a.s.l. (Fig. S2).

The plant-lepidopteran networks decreased in size towards the higher elevations and wet season, although the generally largest network was recorded at 1,100 m a.s.l. in dry season (Fig. 2). The trends in the network characteristics were minor or none, except NODF nestedness. Network connectance slightly increased along the elevational gradient and remained similar between seasons. Q modularity slightly decreased towards the higher elevations and during wet season. Whilst NODF nestedness increased along elevation during dry season and showed an opposite trend during wet season. H_2' specialisation slightly

increased along elevation during wet season, whilst no pattern was observed during dry season (Fig. 3a-e).

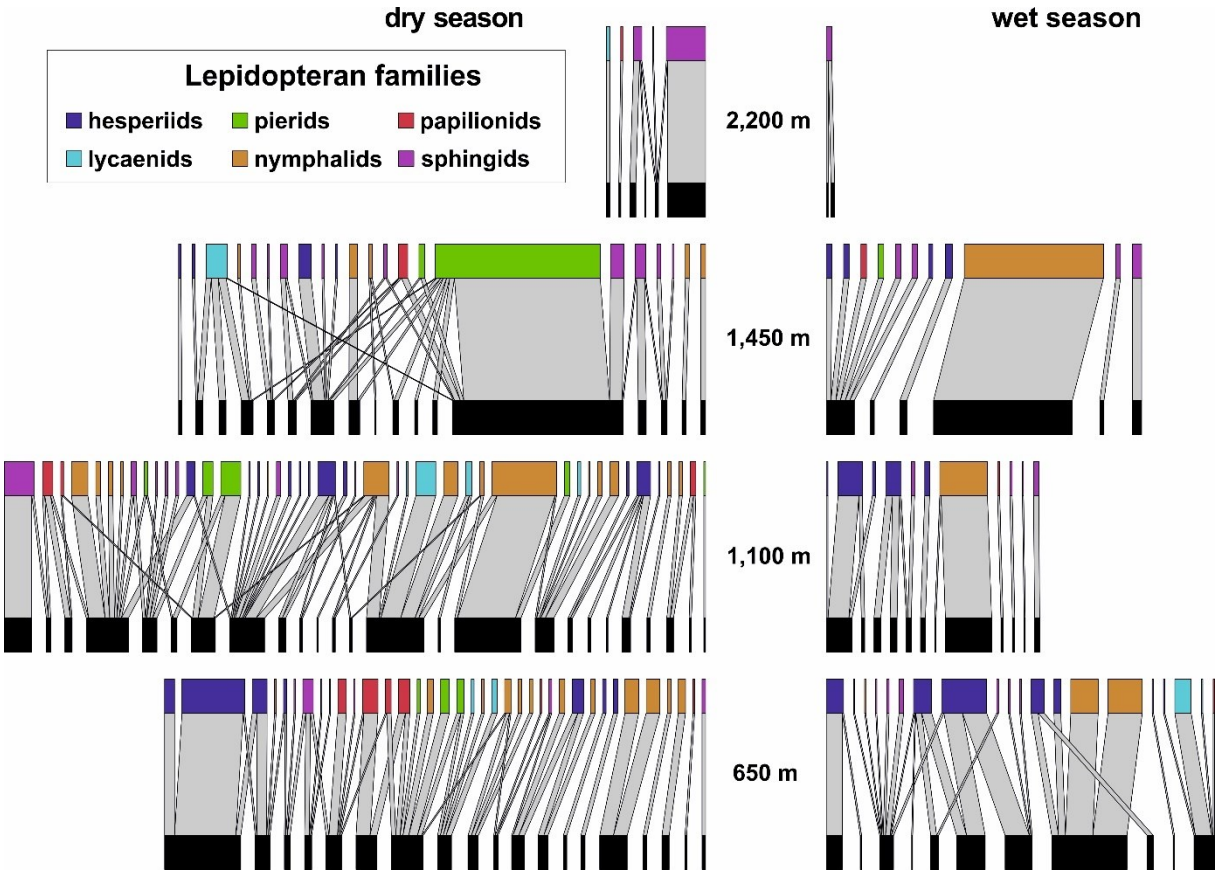

Figure 2. Bipartite networks of plant-lepidopteran interactions along the elevational gradient of Mount Cameroon. The upper nodes visualise flower-visiting lepidopteran species, distinguished by colour for families, whilst the lower nodes represent lepidopteran-visited plant species. The total width of each network approximates their relative size corrected for the sampling effort (visitation frequency per 24hrs of video-recording). The width of individual links (light grey) represents the relative frequency of interactions between visiting lepidopterans and visited plants within each network.

The studied lepidopteran families did not significantly differ in *d'* specialisation (F = 0.865, p = 0.508), nor there was any significant difference in *d'* specialisation between sphingids and all butterflies (F = 0.287, p = 0.593). Model comparisons of the effects of elevation, season, and their interaction showed that the interaction effect of both factors is the most plausible descriptor of the observed patterns in *d'* specialisation (Fig. 3e; Table 2).

Table 2. Comparison of the effects of season, elevation, and their interaction on *d'* specialisation of flower-visiting lepidopterans on Mount Cameroon. LMM with the lepidopteran families as random-effect variable were applied; models with $\Delta AICc \leq 2$ were considered comparable.

All lepidopterans	***residual df***	***residual deviance***	***ΔAICc***	***weight***	***R^2adj***
season	149	7.00	11.1	0	0
elevation	147	6.77	10.3	0.01	0.017
season X elevation	**144**	**6.05**	**0**	**0.99**	**0.102**

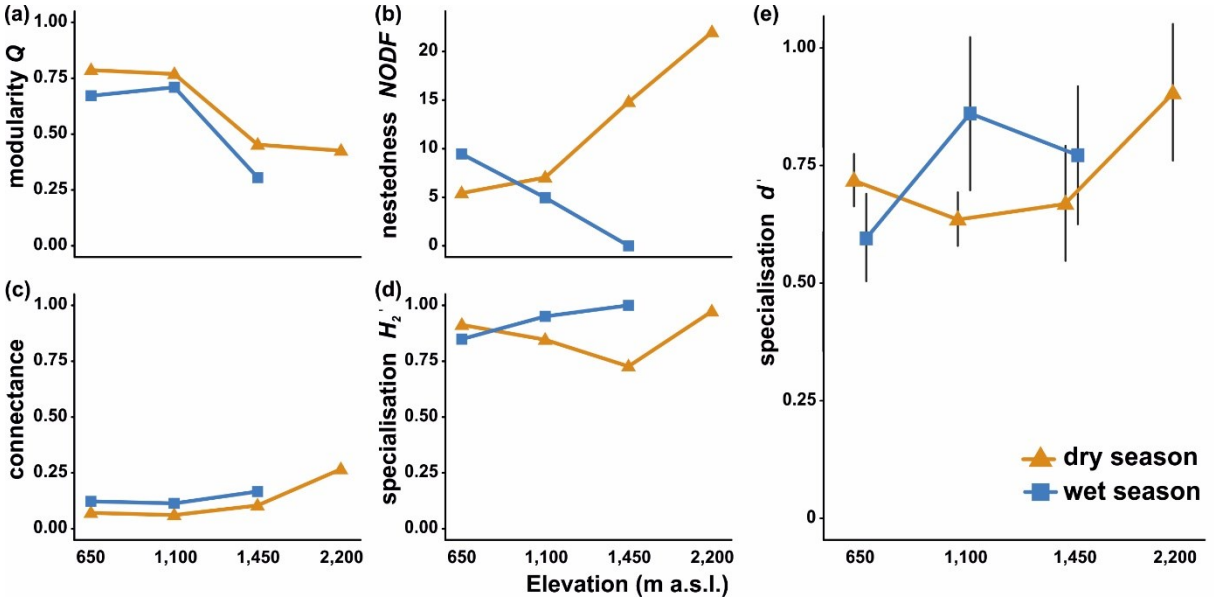

Figure 3. Metrics of plant-lepidopteran networks on Mount Cameroon, comparatively for each elevation and season. The symbols depict arithmetic means in all plots, whilst error bars in (e) represent 95% confidence intervals.

Patterns in lepidopteran morphological traits

Both proboscis and forewing lengths differed significantly among the families (proboscis length: $\chi^2 = 12.15$, $df = 2$, $p = 0.002$; forewing length: $\chi^2 = 31.95$, $df = 2$, $p < 0.001$). Sphingids had on average the longest proboscides, followed by papilionids and hesperiids. Papilionids had on average the longest forewings, followed by sphingids and hesperiids (Table S5). None of the three families showed any significant patterns in their proboscis or forewing lengths along the elevational gradient (Table S6). However, when analysing only the flower-visiting species, elevation became the most plausible descriptor of the U-shaped patterns in their

proboscis and forewing lengths (Table 3; Fig. 4a,b). Even though only three species were measured at the highest elevation, omitting them had no substantial effect on the model strength (Table S7). The proboscis or forewing lengths had no significant effect on *d'* specialisation (proboscis length: $p = 0.38$, $R^2 = 0.16$; forewing length: $p = 0.63$, $R^2 = 0.086$).

Table 3 Linear model comparison of the individual effects of season and elevation, and their interaction, on proboscis and forewing length of lepidopterans on Mount Cameroon. '*res. df*' and '*res. dev.*' represent the residual's degrees of freedom and deviance, respectively.

Proboscis length	***res. df***	***res. dev.***	***ΔAICc***	***weight***	***R²adj***
season	51	73.069	3.2	0.16	0.007
elevation	**49**	**64.940**	**0**	**0.78**	**0.114**
season X elevation	45	58.864	5.2	0.06	0.180
Forewing length					
season	52	15.631	9.4	0.01	0
elevation	**50**	**1.276**	**0**	**0.88**	**0.062**
season X elevation	46	-5.802	4.1	0.11	0.090

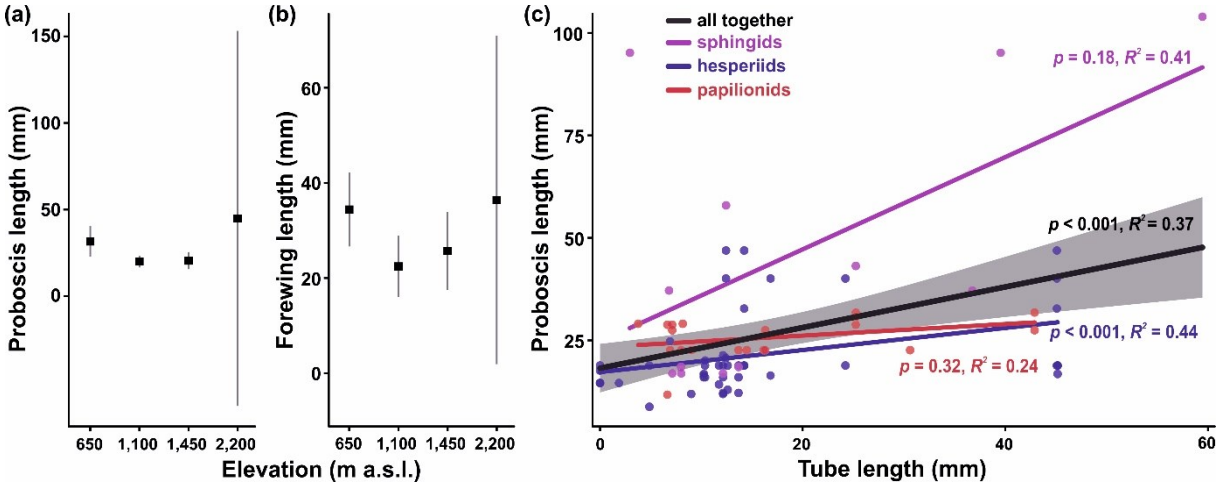

Figure 4. (a) Proboscis and (b) forewing lengths of flower-visiting lepidopterans on Mount Cameroon. Mean values and 95% confidence intervals are visualised. (c) Spearman correlations of lepidopteran proboscis length and corolla tube length of lepidopteran-visited plants. Each data point represents an interaction between a plant species and a lepidopteran species. The black line visualises correlation of

all data (with grey shaded confidence intervals), whilst the coloured lines visualise correlations of individual lepidopteran families.

Lepidopteran preferences to floral traits

Flower-visiting lepidopterans showed significant preferences towards certain floral traits (Fig. 5). Within the complete flowering plant dataset, the selected floral traits explained 25% variability in the visitation frequency (Fig. 5a). The focal families formed three relatively distinct groups. Sphingids preferred sugar-rich, larger and deeper flowers of purple colour. Papilionids, lycaenids and nymphalids preferred orange flowers, whilst hesperiids and pierids did not express any apparent preferences to floral traits. These preferences were mostly consistent with the analysis including only the visited flowers (Fig. 5b), although hesperiids preferred pink actinomorphic flowers. We also found a significantly positive correlation between lepidopteran proboscis length and corolla tube length of lepidopteran-visited flowers (Fig. 4c). However, from the three lepidopteran families, the observed relationship was only significant for hesperiids when analysed separately (Fig. 4c).

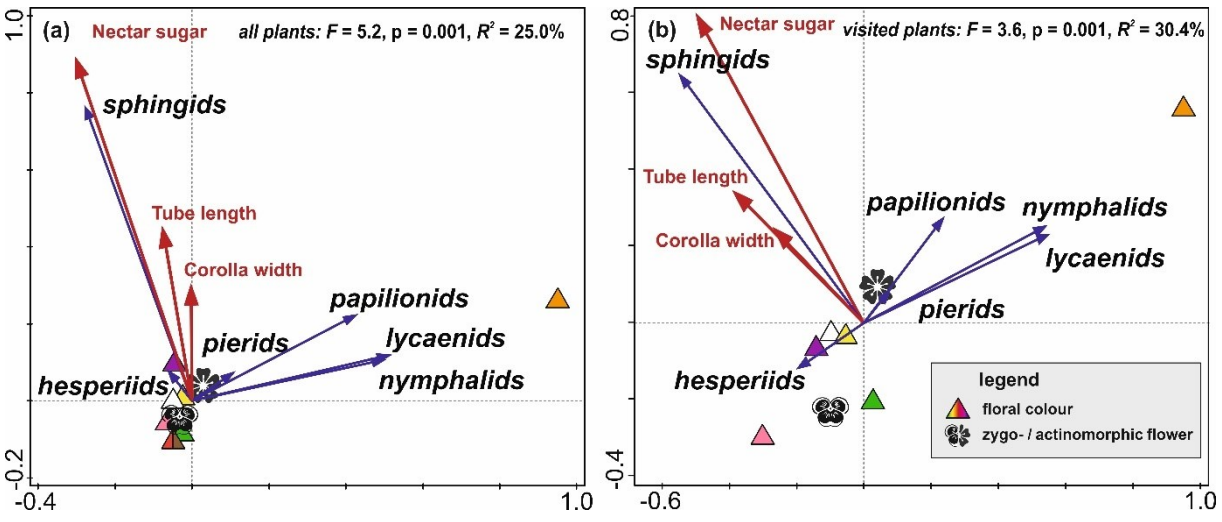

Figure 5. Redundancy analyses (RDA) revealing significant preferences of butterfly and sphingid families (represented by blue arrows) to floral traits (represented by red arrows and various symbols)

on Mount Cameroon. The two RDA models were run for (a) all flowering plant species, and (b) the plant species visited by butterflies or moths.

Discussion

Importance of butterflies and sphingids as pollinators

On Mount Cameroon, butterflies and sphingids generally did not represent the most important pollinators as they collectively made up 4% of the flower-visiting community in tropical rainforests. Their numbers were dwarfed by flower-visiting bees, flies and beetles, representing 44.10%, 25.71% and 11.83% of visits of all recorded plants, respectively (Klomberg *et al.*, 2020). The relative importance of lepidopterans in our uniquely comprehensive Afrotropical networks was even lower than in several partial networks from tropical forests of South-East Asia (Momose *et al.*, 1998; Kato *et al.*, 2008) and the Neotropics (e.g. Ramirez, 1989; Van Dulmen, 2001). In all these studies, flower visitation by lepidopterans was considerable (between 10-20% of all pollinators), although incomparable to bees (between 40-55%). We are not aware of any similar study from the Afrotropical forests.

Even though butterflies and sphingids visited about a third of all flowering plants in the study area, only a few plant species seemed to be primarily pollinated by these groups. Butterflies were the most common visitors of a single plant species, *Scadoxus cinnabarinus*, already known to be butterfly-pollinated (Mertens *et al.*, 2020). In only a few other plant species, butterflies ranked high among all visiting groups (Table S4). Sphingids were not the most common visitors of any recorded plant species, but they ranked second visiting groups of *Anthocleista scandens*. Although plants within this genus have been reported as potentially pollinated by moths or sunbirds (Nsor & Chapman, 2013), its typically widely open chiropterophilous flowers (Weber, Kalko, & Fahr, 2009) do not morphologically fit to sphingophily. However, several other plants commonly visited by butterflies (e.g. *Aframomum*

spp. and *Cordia aurantiaca*) and sphingids (e.g. *Mussaenda tenuiflora* and *Clerodendrum silvanum*) in our study offer morphologically specialised flowers fitting to the lepidoptera-related pollination syndromes (Willmer, 2011). Their efficient pollination by lepidopterans can be expected from other studies of relative or similar plant species from other areas (e.g. Borges et al., 2003; Mizusawa et al., 2014). Several other plants commonly visited by the studied lepidopterans offered rather morphologically generalised inflorescences (e.g. *Distephanus biafrae*, *Melanthera scandens*, and *Crassocephalum montuosum*; all Asteraceae). Plants with such inflorescences were pollinated by butterflies or moths in some cases, although they were typically visited by rich pollinator communities and apparently did not only rely on lepidopterans (Valentin-Silva *et al.*, 2016; Budumajji & Solomon Raju, 2018). Altogether, only a few plant species in our study seemed to depend on pollination by butterflies or sphingids based on the combination of their flowers' morphology and visitation frequency. In conclusion, butterflies and sphingids seem to be relatively less relevant pollinators in tropical forests.

Seasonal patterns of lepidopteran pollination

Pollination networks of butterflies and sphingids strongly differed between the studied seasons on Mount Cameroon. This was surely influenced by a plethora of factors affecting communities of both flower-visiting lepidopterans and flowering plants. The very high inter-seasonal turnover of both butterfly and moth species composition, as well as changes in species richness and abundance, were already reported in detail from Mount Cameroon (Maicher *et al.*, 2018), as well as from other Afrotropical rainforests (e.g. Valtonen et al., 2013). Together with the confirmation of the high species turnover between dry and wet seasons (Fig. S2), this study also revealed seasonal changes in lepidopteran behaviour and networks of their pollination interactions. The general decline of flower-visiting butterflies is most probably connected to the local extreme precipitation during wet season. Adult butterflies and sphingids, as other

large-winged insects, avoid the extreme rainfall on Mount Cameroon (Maicher *et al.*, 2020). Simultaneously, the strong rains and related humidity also affect availability of nectar and its concentration (e.g. Aizen, 2003). The strong seasonality affects the flowering plant communities as well. Whereas many trees, usually offering large amounts of generally accessible nectar, flower in dry season, herbs and shrubs flowering in wet season are not able to replace this nectar production. Such unpublished suggestions were supported by the relatively high turnover of flowering plants between the two seasons (Fig. S2). Moreover plants flowering in the harsh conditions during wet season on Cameroon are often adapted to sunbirds (Bartoš & Janeček, 2014; Janeček, Bartoš, & Njabo, 2015). These could be both causes and consequences of the generally lower diversity and abundance of nectarivorous lepidopterans during wet season.

The prevailingly non-apparent trends in the plant-lepidopteran networks characteristics can be surprising because several other studies of pollination networks along elevation revealed diverse strong patterns (e.g. Ramos-Jiliberto *et al.*, 2010; Classen *et al.*, 2020). Nevertheless, we are not aware about any similar study on pollination by butterflies or sphingids along any tropical elevational gradient for comparison. Therefore, we hypothesise that lepidopteran species are surprisingly specialised for visited flowers, as visible in our relatively less connected and high specialised networks, and in the preferences for distinct floral traits. These preferences only partly change with environmental conditions (Klomberg *et al.*, 2020). However, as discussed above, only a minor part of the visited flowers are specialised for lepidopteran pollination. Therefore, the significant trends in characteristics of the rather generalised plant-lepidopteran networks can hardly be expected. Nevertheless, only more studies of plant-lepidopteran networks from other tropical areas can challenge such hypotheses. We admit that for the only characteristic with a strong trend, nestedness, we do not have any apparent explanation, especially because the trends strongly differed inter-seasonally.

Elevational patterns of lepidopteran pollination

Species richness of butterflies and sphingids showed the 'low-elevation plateau with a mid-peak' (*sensu* McCain & Grytnes, 2010), in accordance with numerous studies of tropical lepidopterans (e.g. Bärtschi et al., 2019; Maicher et al., 2020). The flower visitation frequencies of butterflies mirrored this result, whilst the importance of sphingids in pollination networks, both absolute and relative, increased along elevation. Because we are not aware of any studies on plant-lepidopteran pollination networks along any tropical elevational gradient for comparison, we can hardly speculate if the revealed patterns can be general. However, a study of the *Scadoxus cinnabarinus* pollination system showed peaking diversity and abundance of flower-visiting butterflies at mid-elevations of the plant range (Mertens *et al.*, 2020). Besides numerous factors responsible for the generally high diversity at mid-elevations in many insect groups (e.g. Beck *et al.*, 2017; Bärtschi *et al.*, 2019), the patterns of plant-lepidoptera networks can be discussed in relation to the floral resources for nectarivorous butterflies and sphingids. Although we have no detailed data on the abundance of floral resources along the studied elevational gradient, the local diversity of trees linearly decreased along the gradient (Hořák *et al.*, 2019). However, it remains questionable whether all flowering plants follow this pattern. The opposite trends of sphingid species richness and their importance in networks can be related to the dominance of a few highly mobile species among flower-visiting sphingids in all networks. Whilst their elevational diversity pattern was driven by numerous species with restricted elevational ranges on Mount Cameroon (Maicher *et al.*, 2020), most of the identified sphingids in our networks were generalised long-distance vagrants (Table S1). Considering the generally small sizes of the plant-sphingid networks and the relatively smaller elevational variability, the revealed patterns could be caused by more or less random visitation by these mobile generalists.

Traits in plant-lepidopterans networks

We found no evidence that longer proboscides or forewings of butterflies and sphingids elicited differences in the flower visitation behaviour, apart from the correlation between lengths of proboscis and floral tubes of flowers visited by hesperiids. The similar proboscis-tube relationship was already found in other studies (Corbet, 2000; Bloch & Erhardt, 2008; Martins & Johnson, 2013; Johnson *et al.*, 2017), although such studies often found larger butterflies visiting larger flowers as well (e.g. Corbet, 2000; Tiple et al., 2009). Nevertheless, although preferring flowers with longer tubes, the long-proboscid lepidopterans were not more specialised in the meaning of their food-niche breadth (relative number of visited plant species) in our study. Therefore, we assume that even these morphologically specialised lepidopteran species were rather looking for any available resources in longer or deeper flowers which are more likely to be unreachable by other floral visitors, rather than being specialised for a few co-evolved plant species (cf. Martins & Johnson, 2013; Johnson et al., 2017).

Although we found no significant patterns among the morphological traits of lepidopterans along elevation when analysing all captured butterflies and sphingids, the lengths of forewing and proboscides showed U-shaped pattern when the analyses were restricted to the flower-visiting species. We expect that such patterns can be covered by other mechanisms when analysing complete lepidopteran community, including species with adults feeding on other resources than flowers. The increase of lepidopteran size towards the higher elevations was repeatedly reported and explained mostly by the need of larger area for basking in colder environments (e.g. Brehm et al. 2019). Nevertheless, the relatively larger bodies of lepidopterans in the lowest elevation seems surprising and difficult to explain.

Generally, preferences of butterflies and sphingids to the visited flowers were driven by floral colour, size, and nectar-sugar production in our study. This corroborates to numerous other studies (e.g. Tiple et al., 2009; Willmer, 2011). Moreover, the floral preferences neatly

separated nocturnal sphingids from diurnal butterflies, as proposed by the pollination syndrome hypothesis (Willmer, 2011). Concurrent with other studies (Kaczorowski *et al.*, 2012; Johnson *et al.*, 2017), sphingids preferred longer and nectar-rich flowers on Mount Cameroon. Opposite to the sphingophilous syndrome (Willmer, 2011), they did not seem to prefer white flowers. Papilionids, nymphalids and pierids preferred orange flowers, the other floral traits were much less relevant. Strong preferences to floral colours were already shown for butterflies (e.g. Ômura & Honda, 2005; Pohl et al., 2011), and orange flowers are typical for the psychophilous plants (Willmer, 2011). Pierids and hesperiids expressed little to no preferences to any floral traits in this study. Such differences among butterfly families were already observed (Yurtsever *et al.*, 2010; Pohl *et al.*, 2011). Our detailed taxon-specific approach uncovered some of the limitations of the pollination syndrome hypothesis, especially that different traits can differ in their importance among particular syndromes, and that even individual subgroups of the single pollinator group can differ in their preferences (cf. Dellinger, 2020; Klomberg et al., 2020).

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Authors' contributions

RT, ŠJ and JEJM conceived the ideas and designed methodology; JEJM, ŠJ, YK, INK and RT collected the plant-lepidopteran interactions data; JEJM, VM, SzS, RT, SD, PP, INK, LB and TP collected, identified and/or measured lepidopterans; ŠJ, YK and INK measured floral traits; JEJM and LB analysed data; JEJM and RT led the manuscript writing. All authors contributed to the drafts and approved for publication.

Data Availability Statement

Data available via the Zenodo repository (*doi will be provided after acceptance*).

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Supplementary material

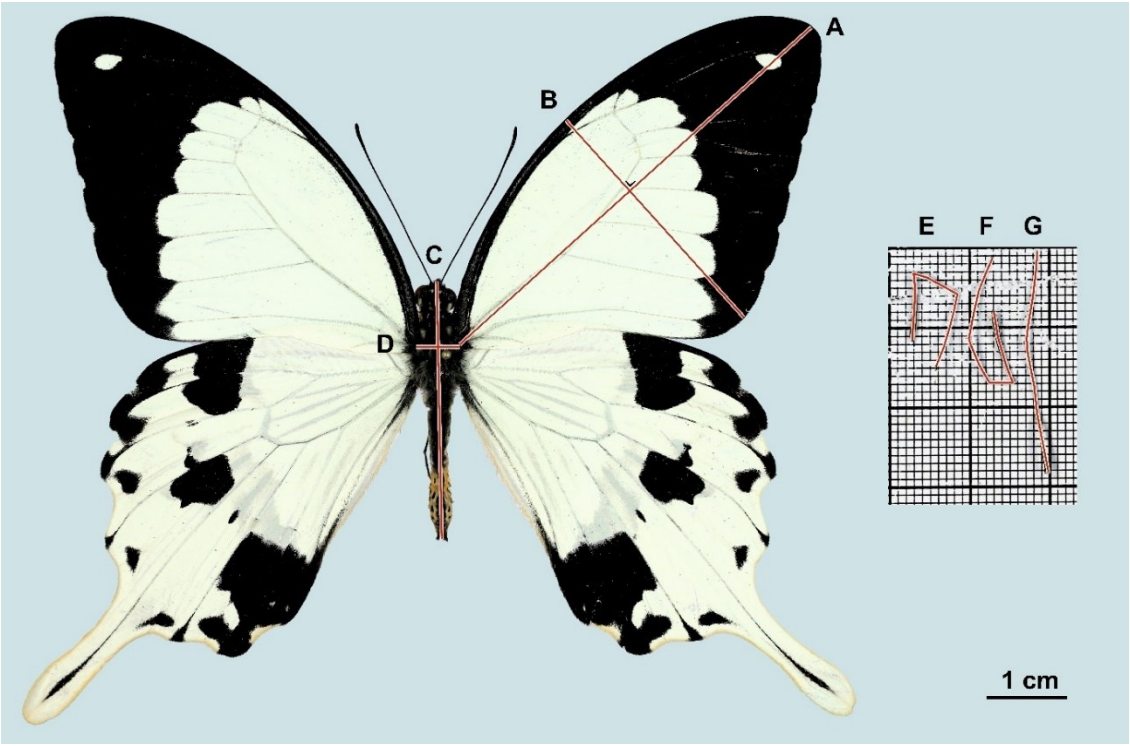

Figure S1. Illustration of the measured traits of flower-visiting butterflies and sphingids on Mount Cameroon. (A) Forewing length: from a wing base to a wingtip (defined as point where the tangent of a wing edge is perpendicular to the measure line). (B) Forewing width: perpendicular to the forewing length measure line and positioned so that the tangent of the outer wing margin is perpendicular to the measure line. (C) Body length: from the top of a head (excluding mouthparts) to the end of an abdomen (excluding genital valves). (D) Body width: measured where forewings attach to a thorax. (E-G)

Lengths of fore-, mid-, and hindleg, respectively: from a base of tibia to the last tarsus (excluding tarsal claw), measured by a segmented line.

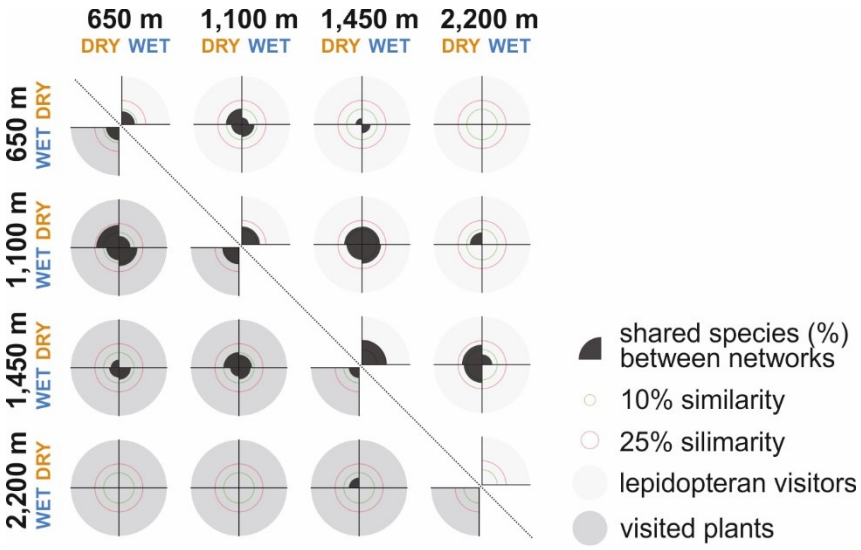

Figure S2. Turnover of lepidopteran and plant species in the studied plant-lepidoptera pollination networks on Mount Cameroon, visualised as proportions of the shared butterfly and sphingid visitor species and visited plant species among particular elevations and seasons.

Table S1. List of all flower-visiting butterflies and sphingids identified from the video recordings on Mount Cameroon.

Hesperiidae	***Nymphalidae***	***Papilionidae***
Acleros bibundica	*Acraea alcinoe*	*Graphium policenes*
Andronymus magma	*Acraea bonasia*	*Papilio charopus*
Andronymus sp.1	*Acraea elongate*	*Papilio cyproeofila*
Apallaga alluaudi	*Acraea epaea*	*Papilio dardanus*
Apallaga intermixtus	*Acraea judutta*	*Papilio hesperus*
Apallaga meditrina	*Acraea lycoa*	*Papilio menestheus*
Apallaga mona	*Acraea penelope*	*Papilio zenobia*
Bettonula bettoni	*Acraea pharsalus*	*Papilio zoroastres*
Celaenorrhinus dargei	*Acraea rogersi*	
Ceratrichia clara	*Acraea tellus*	**Sphingids**
Ceratrichia fako	*Acraea umbra*	*Agrius convolvuli*
Ceratrichia flava	*Amauris damocles*	*Centroctena rutherfordi*
Coeliades chalybe	*Amauris echeria*	*Hippotion osiris*
Coeliades forestan	*Bebearia tentyris*	*Hippotion* sp.1
Coeliades hanno	*Bicyclus anisops*	*Hippotion* sp.2
Coeliades libeon	*Bicyclus sciathis*	*Hippotion* sp.3
Eagris decastigma	*Cymothoe beckeri*	*Macroglossinae* sp.1
Gorgyra sp.1	*Cymothoe consanguis*	*Macroglossinae* sp.2
Leona lissa	*Cymothoe herminia*	*Macroglossinae* sp.3
Meza sp.1	*Cymothoe indamora*	*Macroglossinae* sp.4
Osmodes lux	*Cymothoe sangaris*	*Macroglossinae* sp.5
Osmodes thora	*Cymothoe weymeri*	*Macroglossinae* sp.6
Paracleros sp.1	*Euphaedra hewitsoni*	*Macroglossinae* sp.7
Pardaleodes tibullus	*Euphaedra losinga*	*Macroglossinae* sp.8
Paronymus xanthias	*Euphaedra* sp.1	*Macroglossum trochilus*
Semalea sp.1	*Euphaedra temerraria*	*Nephele accentifera*
Tagiades flesus	*Hypolimnas salmacis*	*Nephele* sp.1
	Junonia terea	*Nephele* sp.2
Lycaenidae	*Phalanta eurytis*	*Nephele* sp.3
Anthene definita	*Precis milonia*	*Nephele* sp.4
Cacyreus cf. *lingeus*	*Vanessula milca*	*Temnora iapygoides*
Euchrysops malathana		*Temnora* sp.1
Hypolycaena sp.1	***Pieridae***	*Theretra orpheus*
Neurypexina lamprocles	*Appias* cf. *sabina*	*Theretra* sp.1
Thermoniphas alberici	*Leptosia alcesta*	*Theretra* sp.2
Zizula hylax	*Leptosia* cf. *hybrida*	*Xanthopan morganii*
	Leptosia nupta	

Table S2. Intercorrelation matrix of the measured traits of flower-visiting butterflies and sphingids on Mount Cameroon. After application of Bonferroni correction (39 analyses), only $p < 0.001$ were considered significant, these are highlighted by **bold**. All correlations were positive.

	Weight	**Proboscis length**	**Forewing length**	**Forewing width**	**Body length**	**Body width**	**Foreleg length**	**Midleg length**
Proboscis length	$R^2 = 0.46$, $p = 0.007$							
Forewing length	**$R^2 = 0.89$, $p < 0.001$**	$R^2 = 0.42$, $p = 0.014$						
Forewing width	**$R^2 = 0.74$, $p < 0.001$**	$R^2 = 0.36$, $p = 0.037$	**$R^2 = 0.93$, $p < 0.001$**					
Body length	**$R^2 = 0.97$, $p < 0.001$**	**$R^2 = 0.56$, $p = 0.001$**	**$R^2 = 0.88$, $p < 0.001$**	**$R^2 = 0.74$, $p < 0.001$**				
Body width	**$R^2 = 0.96$, $p < 0.001$**	$R^2 = 0.49$, $p = 0.004$	**$R^2 = 0.84$, $p < 0.001$**	**$R^2 = 0.68$, $p < 0.001$**	**$R^2 = 0.98$, $p < 0.001$**			
Foreleg length	**$R^2 = 0.89$, $p < 0.001$**	**$R^2 = 0.58$, $p < 0.001$**	**$R^2 = 0.94$, $p < 0.001$**	**$R^2 = 0.87$, $p < 0.001$**	**$R^2 = 0.92$, $p < 0.001$**	**$R^2 = 0.86$, $p < 0.001$**		
Midleg length	**$R^2 = 0.90$, $p < 0.001$**	$R^2 = 0.52$, $p = 0.002$	**$R^2 = 0.94$, $p < 0.001$**	**$R^2 = 0.83$, $p < 0.001$**	**$R^2 = 0.92$, $p < 0.001$**	**$R^2 = 0.89$, $p < 0.001$**	**$R^2 = 0.92$, $p < 0.001$**	
Hindleg length	**$R^2 = 0.94$, $p < 0.001$**	$R^2 = 0.41$, $p = 0.019$	**$R^2 = 0.92$, $p < 0.001$**	**$R^2 = 0.82$, $p < 0.001$**	**$R^2 = 0.93$, $p < 0.001$**	**$R^2 = 0.88$, $p < 0.001$**	**$R^2 = 0.93$, $p < 0.001$**	**$R^2 = 0.88$, $p < 0.001$**

Table S3. Summary of butterflies and sphingids visiting of flowers / touching its reproductive organs at each sampling elevation and season on Mount Cameroon.

Elevation (m a.s.l.)	**season**	**butterflies**					**sphingids**	**all lepidopterans**
		hesperiids	**lycaenids**	**nymphalids**	**papilionids**	**pierids**		
650	**dry**	69 / 65	3 / 3	22 / 21	19 / 19	12 / 12	13 / 13	138 / 133
1,100	**dry**	33 / 24	17 / 17	76 / 75	10 / 10	26 / 26	17 / 17	179 / 169
1,450	**dry**	11 / 2	10 / 10	11 / 11	7 / 7	141 / 137	29 / 28	209 / 195
2,200	**dry**	/	1 / 1	/	3 / 3	1 / 1	23 / 23	28 / 28
650	**wet**	60 / 57	13 / 13	10 / 10	1 / 1	/	10 / 10	94 / 91
1,100	**wet**	25 / 23	/	20 / 20	1 / 1	/	6 / 6	52 / 50
1,450	**wet**	4 / 4	/	20 / 20	1 / 1	1 / 1	4 / 4	30 / 30
2,200	**wet**	/	/	/	/	/	4 / 4	4 / 4
Total		**628 / 595**					**106 / 105**	**734 / 700**

Table S4. Plant species most visited by butterflies (top 10) and hawkmoths (top 5) on Mount Cameroon, as well as the rank of butterflies/sphingids among all functional groups of flower visitors (Klomberg et al. 2020).

butterflies			
plant family	**plant species**	**No. visits**	**rank**
Asteraceae	*Distephanus biafrae*	136	2
Zingiberaceae	*Aframomum* sp. 'purple'	67	3
Compositae	*Melanthera scandens*	63	2
Amaryllidaceae	*Scadoxus cinnabarinus*	39	1
Balsaminaceae	*Impatiens macroptera*	27	3
Vitaceae	*Cissus oreophylla*	24	3
Apocynaceae	*Tabernaemontana ventricosa*	22	5
Asteraceae	*Crassocephalum montuosum*	22	5
Boraginaceae	*Cordia aurantiaca*	17	2
Balsaminaceae	*Impatiens mannii*	16	3

sphingids			
plant family	**plant species**	**No. visits**	**rank**
Gentianaceae	*Anthocleista scandens*	15	2
Asteraceae	*Distephanus biafrae*	13	6
Rubiaceae	*Mussaenda tenuiflora*	11	3
Apocynaceae	*Tabernaemontana ventricosa*	8	7
Lamiaceae	*Clerodendrum silvanum*	7	4

Table S5. Summary of average trait values of butterflies and sphingids on Mount Cameroon.

	Proboscis length (cm)		**Forewing length (cm)**	
	mean	***sd***	***mean***	***sd***
papilionids	2.601	0.695	5.500	0.812
hesperiids	2.030	1.076	1.860	0.362
sphingids	5.324	3.473	3.344	1.299

Table S6. Results of linear models analysing effects of elevation on proboscis and forewing lengths of all butterflies and sphingids captured on Mount Cameroon.

	Proboscis length				**Forewing length**			
	df	***F***	***p***	***R²adj***	***df***	***F***	***p***	***R²adj***
all taxa	6	1.324	0.247	0.030	6	0.847	0.535	0.004
sphingids	6	0.626	0.709	0.052	6	1.130	0.356	0.088
hesperiids	6	0.720	0.634	0.033	6	0.925	0.480	0.039
papilionids	6	0.226	0.966	0.028	6	0.553	0.765	0.065

Table S7. Comparison of linear mixed-effect models (with family as random-effect variable) analysing effects of season, elevation, and their interaction on proboscis and forewing lengths of butterflies and sphingids visiting flowers on Mount Cameroon. Models with $\Delta AICc \leq 2$ from the most plausible model were considered as comparable.

	residual df	***residual deviance***	***ΔAICc***	***weight***	***R²adj***
Proboscis length					
Season	47	64.062	6.5	0.04	0
Elevation	**46**	**53.372**	**0**	**0.96**	**0.124**
season X elevation	43	52.951	11.6	0	0.119
Forewing length					
Season	48	13.718	8.4	0.02	0
Elevation	**47**	**-0.486**	**0**	**0.98**	**0.046**
season X elevation	44	-4.651	11.7	0	0.119

CHAPTER III

Chmel K, Ewoma FL, Uceda Gomez G, **Klomberg Y**, Mertens JEJ, Tropek R, Janeček Š (Submitted manuscript). Bird pollination syndrome works as the plant's adaptation to ornithophily, while nectarivorous birds do not seem to care.

YK was strongly involved in the fieldwork and processing of the "insect data". YK oversaw measuring floral traits and maintaining the trait database. Furthermore, YK contributed to writing of the manuscript.

Bird pollination syndrome works as the plant's adaptation to ornithophily, while nectarivorous birds do not seem to care.

Submitted to Oikos (18-10-2020)

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Key words: nectarivorous birds, floral choice, nectar resource, floral traits, pollination, Mount Cameroon National Park

Abstract

Many tropical plants are pollinated by birds and several bird phylogenetical lineages specialised to a nectar diet. The long assumed intimate ecological and evolutionary relationship between ornithophilous plants and phenotypically specialised nectarivorous birds has nevertheless been questioned in recent decades, where such plant-pollinator interactions have been shown to be highly generalised.

In our study we analysed two extensive interaction datasets both collected on Mt. Cameroon: sunbird-flower and insect-flower data. We tested if 1) insects and birds interact with distinct groups of plants; 2) plants with a typical set of ornithophilous floral traits, i.e. bird pollination syndrome, interact mainly with birds; 3) birds favour ornithophilous plants; and 4) if and how the individual floral traits and plant level nectar production predict the bird visitation.

The plant community did not exhibit a strong separation into bird-visited plants and insects hosting plants, but rather a continuum of strategies where plants, frequently or rarely, interact with both insects and birds. We confirmed the validity of the bird pollination syndrome hypothesis from the standpoint of plants. The ornithophilous plants were visited by birds at a higher rate and mostly hosted a lower frequency of visiting insects. However, from the bird perspective, the ornithophilous plants were not more attractive compared to non-ornithophilous plants for nectar-feeding birds. Nectar production per plant individual was a better predictor of bird visitation than any other floral trait traditionally related to the bird pollination syndrome. Our study did not show a two-sided specialised but highly asymmetrical relationship between ornithophilous plants and nectarivorous birds.

Introduction

Most tropical plants rely on animals for pollination (Ollerton, Winfree, & Tarrant, 2011) and floral food rewards are important parts of the diet for many animal groups. Consequently, in tropical rainforests, approximately 98% of flowering plants are estimated to be animal-pollinated (Bawa, 1990). Although most of them depend on insect pollinators (Proctor, Yeo, & Lack, 1996), vertebrates, particularly birds and mammals, also represent important pollen vectors in tropical pollination networks (Fleming & Muchhala, 2008). While there are a number of nectar feeding bird species across different families (Proctor *et al.*, 1996), we recognise three families of flower specialists globally: hummingbirds (Trochilidae) in the Neotropics, sunbirds (Nectariniidae) in the Old World, and honey-eaters (Meliphagidae) in Australasia (Fleming & Muchhala, 2008).

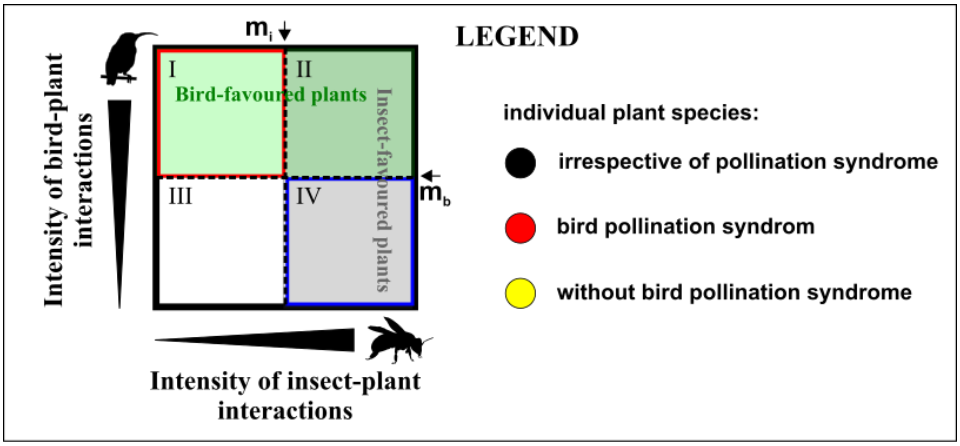
The concept of mutualistic evolutionary adaptations in plant-pollinator interactions was first put forth by Darwin as his „evolutionary race“ (Darwin, 1862). Among others, this concept includes, step by step co-evolution where both partners (or partner groups) continuously increase the level of their co-adaptations. Gradually producing a system characterised by high specialisation on both sides. Pollination syndromes are a central concept of plant adaptation, which hypothesises that plants pollinated by the same pollinator group share similar floral traits via convergent evolution (Faegri & van der Pijl, 1979). Ornithophilous flowers tend to be red or orange, tubular with copious nectar, and lack characters associated with other syndromes, such as a strong scent or presence of nectar guides (Faegri & van der Pijl, 1979; Fenster *et al.*, 2004, 2015; Cronk & Ojeda, 2008). In parallel, Stiles (1981) defined the “syndrome of anthophily“ for specialised nectarivorous birds, which includes morphological, physiological, and behavioural adaptations of birds related to nectarivory.

In recent decades however, community-wide studies have demonstrated that the ecological reality and co-evolution processes can be much more complicated, and that the close

relationships in food pollination systems are rather exceptional (Waser *et al.*, 1996). For example, specialised nectarivorous birds are frequent visitors of non-ornithophilous plants (Araujo & Sazima, 2003; Maruyama *et al.*, 2013), where they may act as thieves (Rocca & Sazima, 2006; Janeček *et al.*, 2007; Padyšáková *et al.*, 2013), or in other cases also as co-pollinators (Wolff, Braun, & Liede, 2003; Freitas, Galetto, & Sazima, 2006). Conversely, ornithophilous flowers may also host visitors other than birds (e.g. bumblebees: Pleasants & Waser, 1985; Urcelay, Morales, & Chalcoff, 2006; bees: Schmid *et al.*, 2011). Additionally, the applicability of the bird pollination syndrome differs regionally (Brown & Hopkins, 1995; Fleming & Muchhala, 2008) or along environmental gradients, such as elevation (Araujo & Sazima, 2003; Dalsgaard *et al.*, 2011; Partida-Lara *et al.*, 2018) and precipitation (Dalsgaard *et al.*, 2011; Maruyama *et al.*, 2013). Finally, we must acknowledge that a proportion of plants are adapted to a generalised pollination system, making phenotypic-based predictions of its primary pollinator impossible (Waser *et al.*, 1996; Fenster *et al.*, 2004; Ollerton *et al.*, 2009; Dellinger, 2020).

The pollination syndrome hypothesis, together with the coevolutionary theory, suggests that pollinators have innate preferences for distinct floral traits. Such an assumption, however, only reflects the plant perspective. Although the most efficient pollinators may truly coincide with the defined pollination syndrome (Rosas-Guerrero *et al.*, 2014), from the pollinator perspective, floral choice may likely be more simple with only the quantity and quality of food rewards determining pollinator flower selection (Waser, 1983; Pleasants & Waser, 1985; Stromberg & Johnsen, 1990; Essenberg, 2012; Schmid *et al.*, 2016). This resource-motivated selection has seen indirect support. Studies have shown that a substantial proportion of flowers are pollinated by several different pollinators, and that pollinators visit flowers of multiple plant species often belonging to different pollination syndromes (Herrera, 1996; Waser *et al.*, 1996).

In this study, we test the validity of the bird pollination syndrome hypothesis and explore the flower-bird relationships on Mt. Cameroon (West/Central Africa). Our analyses were performed in two complementary ways: First, we used a simple conceptual model to test: 1) if birds and insects prefer distinct groups of plants (Fig. 1a); 2) whether plants with bird pollination syndromes were favoured by birds, but avoided by insects (Fig. 1b); and 3) if birds feed mainly on the ornithophilous plants (Fig. 1c). Second, we tested if 4) any floral traits related to the bird pollination syndrome predicted bird visitation, and 5) whether these traits were better predictors than the nectar production of the whole plant individual.



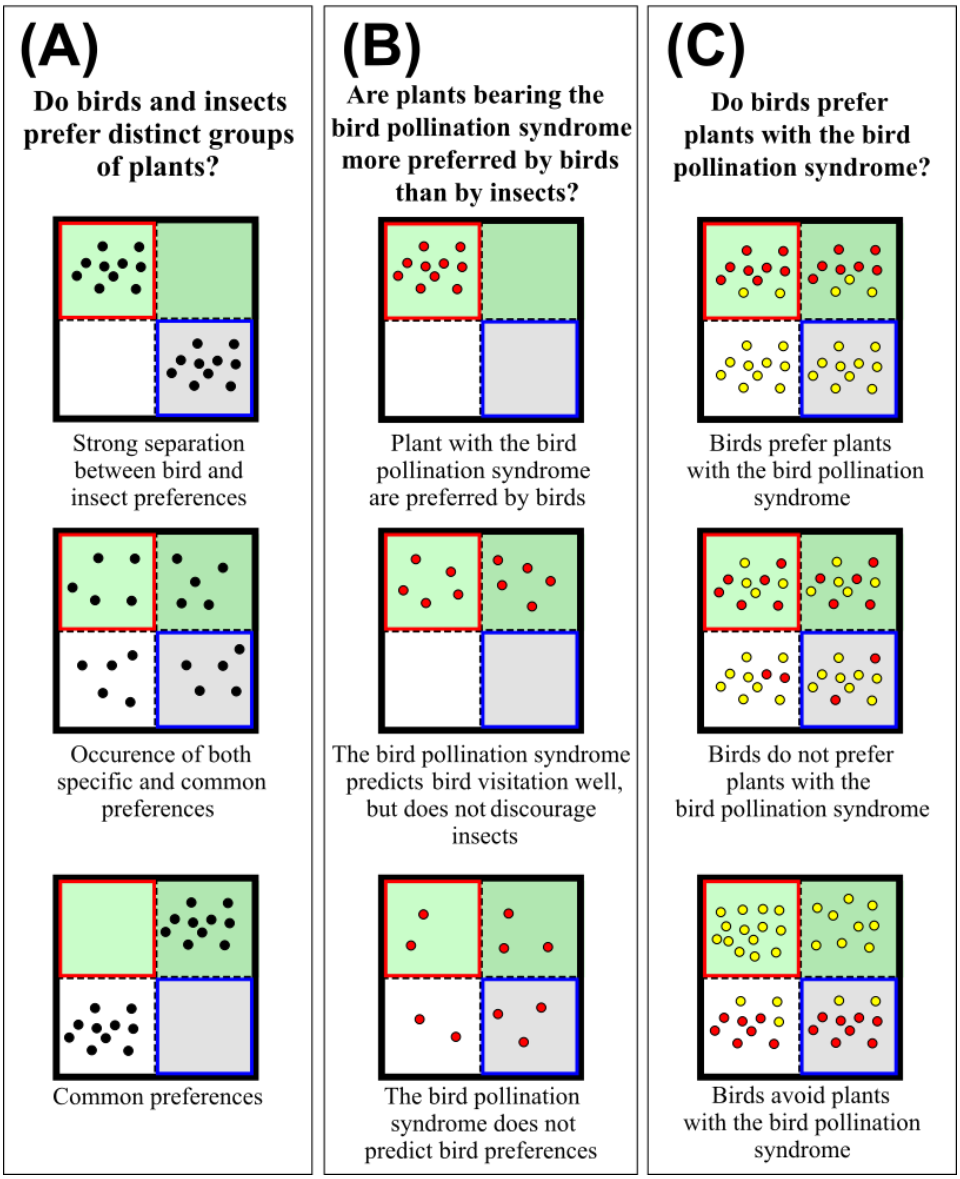


Figure 1. Conceptual model where plant species are organised in the space defined by the axes of bird-plant and insect-plant interaction intensity: I/ a bird-plant interaction zone with a high intensity of bird-

plant interactions (red border); II/ a zone with a high intensity of both bird and insect interactions; III/ a zone with low intensity interactions by both pollinator groups; and IV/ insect-plant interaction zone with a high intensity of insect-plant interactions (blue border). $\mathbf{m_i}$ – median of the intensity of insect-plant interactions dividing the space for the insect preferred (zones II and IV) and insect non-preferred (zones I and III) plants; $\mathbf{m_b}$ – median of the intensity of bird-plant interactions dividing the space to bird preferred (zones I and II) and bird non-preferred (zones III and IV) plants. A, B and C show possible scenarios related to our target questions.

Methods

Study site

All datasets were collected in tropical rainforests on the south-western slope of Mt. Cameroon (Cameroon), the highest mountain of West Africa. On this slope, primary forests are preserved from approximately 400 m a.s.l. up to the treeline at approximately 2,200 m a.s.l. (Cable & Cheek, 1998). The climate of Mt. Cameroon comprises of a period of heavy rains, which occur between June and October, and a dry season which extends from November to May, with an annual rainfall usually exceeding 10,000 mm (Lefevre, 1967; Maicher *et al.*, 2020). The data was collected at four sites distributed along the elevational gradient of the mountain: 1) Drink Garri (650 m a.s.l.) – lowland tropical rainforest with abundant trees from the Caesalpiniaceae family, 2) Plantecam (1,100 m a.s.l.) – mid- elevation forest partly disturbed by elephants, 3) Crater Lake (1,450 m a.s.l.) – submontane forest strongly disturbed by elephants, 4) Mann's Spring (2,200 m a.s.l.) – mountain forest close to the treeline (Maicher *et al.*, 2020). At each site, we collected data both in the wet and dry season. The sampling included vegetation surveys along six 200 x 10 m permanent transects per site, along which we estimated the abundance of flowering plants, together with numbers of flowers per plant individual. Transects were situated at least 100 m apart, so as to account for environmental heterogeneity at each locality.

Floral traits

Based on Faegri and van der Pijl (1979) and Ollerton *et al.* (2009), we selected six floral traits related to the bird pollination syndrome, including floral size (averaged in asymmetric flowers), tube length, odour (weak–no *vs* moderate–strong), colour, nectar guides (presence *vs* absence), and nectar sugar production [mg] per flower. Additionally, we calculated the resource quantity by multiplying the latter trait with the number of flowers per plant, i.e. average nectar production per individual of each species. All measured trait values of the studied plant species are listed in Supplementary Material Table S1. The morphological floral trait measurements were done using a digital calliper. Ideally, a minimum of five replicates of each plant species were measured. If sufficient replicates were not found within the transects, we broadened the search area to the adjoining vegetation. To compare the nectar production of individual plant species, we covered the flowers with mesh bags for a 24-hour period before the nectar measurement. If nectar was abundant, it was extracted and its volume was measured using a Hamilton syringe or capillary tube following Bartoš *et al.* (2012). The nectar concentration was then measured with a Pal-1 (Atago co.) pocket refractometer. Subsequently, the amount of sugar per flower was calculated based on the nectar volume and its concentration. Flowers which produced low volumes of nectar were washed with distilled water which was then collected in an Eppendorf tube. Later in the field camp, we added ethanol to achieve a 50% concentration and boiled the solution to avoid any possible enzymatic degradation during transport to the lab (Chlumská, Janeček, & Doležal, 2014). In the lab, we dried the samples, transferred them into constant volume, and the sugar amounts were measured using the Ionic Chromatograph ICS-3000 (Dionex) with an electrochemical detector and CarboPac PA1 column. Due to their rarity or inaccessibility, we were not able to collect values of sugar production for all plant species that flowered at the study sites and these were therefore omitted from the dataset. The exception was *Phragmanthera kamerunensis*. Because this species

represents an important nectar resource for bird visitors (Janeček in prep.), we used the values of its sugar content (average value in closed flowers) from Gill and Wolf (1975) who explored the energetics of a similar East African species *Phragmanthera dshallensis*.

Based on the traditional definitions of syndromes (Faegri & van der Pijl, 1979; Fenster *et al.*, 2004; Cronk & Ojeda, 2008), we considered flowers of plants to fall within the bird pollination syndrome if they: 1) provided sufficient nectar reward (>0.3 µl per flower); 2) did not produce a strong odour; 3) had a long wavelength, i.e. orange/red colour; 4) were of tubular shape (tube length >1 cm); 5) had tubes or spurs too narrow for nectarivorous birds; 6) had no nectar guides; and 7) effectively placed pollen on the body of birds during visitation.

Observation of insect-plant interactions, dataset 1

The first dataset was collected during four expeditions between 2016–2018. Animal-plant interactions were collected using security cameras (VIVOTEK IB8367T with IR night vision). Up to five specimens of each plant species were recorded, each for 24-h of continuous recording, resulting in 1115 individual recordings from 211 plant species. Floral visitors were found in the video material by either manual watching or using the automatic movement detecting software MotionMeerkat (Weinstein, 2015). For calculation of visitor frequency per hour, we included only insect visitors that made contact with plant reproductive organs (similar to Biella *et al.*, 2019). Among a selection of the most common pollinator groups, we found 13170 insect-plant interactions, with the majority of visits by bees (26.50%; excluding wasps, carpenter bees and honeybees), followed by honeybees (24.84%), hoverflies (15.96%) and moths (10.68%; excluding hawkmoths). The data collection prioritised insect-plant interactions, since the cameras were placed close to the plants to enable the identification of insect morphospecies and to record night visitors using IR night vision. Although the dataset includes other types of visitors as well, such as birds or mammals, the sample size was

relatively low. Particularly for rarer bird-plant interactions too low to consider it robust. This data was therefore used primarily for insect-plant interactions, with the addition of a more complex dataset on bird-plant interactions (see dataset 2 below).

Observation of bird-plant interactions, dataset 2

The second dataset was collected between 2018–2020 at the same study sites as dataset 1. The observational unit was a plant individual. For lianas, where the definition of an individual can be complicated, we distinguished flowering parts which were not seemingly connected as separate units. We observed flowering plants that we considered potentially bird-pollinated and met the following criteria: 1) birds had been observed to feed on the flowers during our long-term research on Mt. Cameroon, or 2) its nectar volume in a flower covered for 24 hrs was higher than 0.3 µl/per flower (Unpublished data); and 3) it flowered at least in three replications along the six transects per study site, or it occurred less frequently within the transects but flowered commonly in the vicinity. Bird-plant interactions (visits during which birds fed on nectar) were observed using two complementary methods. For the observation of herbs and shrubs, we used the same security cameras to record 10 individuals of each plant species. Each plant individual was recorded for two consecutive days (from 6:00 till 18:00 only). Nevertheless, the total recording times for individual plants slightly differed (Table S1), because some species were difficult to find, or due to logistic and/or technical problems which sometimes occurred in the harsh weather on Mt. Cameroon. Finally, the mean length of the video observation was 152 hrs. per plant species. The recorded floral visitors were identified in the same way as for dataset 1.

The second method, conducted simultaneously, was personal observation of trees and larger shrubs, where cameras did not have the capacity to record visits on all flowers at the same time. We aimed to observe eight individuals per plant species, each of them for eight

hours equally distributed during the daytime (from 6:00 till 18:00 only). Because we did not find a sufficient number of individuals of some plant species, the mean length of observation was 68 hrs per species (Table S1). High trees were usually observed from a neighbouring tree, reached by the one-rope climbing technique. In total, we recorded 9,473 bird-plant interactions in dataset 2, i.e. occasions where birds fed on nectar, for the four sites during both dry and wet seasons. 22 species of birds were observed feeding on nectar, with sunbirds (Nectarinidae) being the most prominent visitors (12 species, 90.0% of all visits, Table S2).

Merging of datasets: comparison of bird and insect visitation frequency

We considered 88 plant species which were found in both datasets, i.e. the same species were sampled within dataset 1 and dataset 2. Because both datasets were obtained during different expeditions and using slightly different methodological approaches (see above), we calculated the relative attractiveness of individual plant species for birds and for insects separately. The plants were ranked from the most to least visited species based on bird or insect visitation frequencies (i.e. the average number of individual visitors per hour of observation). For the purpose of comparing bird and insect visitation, we averaged the visitation frequencies for each plant species that occurred at several sites or flowered during both dry and wet seasons. The plant species with no recorded visits were ranked as zero.

Merging of datasets: predictors of bird visitation frequency

Although not all plants were observed in dataset 2 (bird-plant interactions), we were able to provide empirical evidence whether the omitted plant species were visited by birds using dataset 1 (insect-plant interactions). We derived the frequencies of bird visits from dataset 1 for 41 plant species that were flowering, but were omitted during the work on dataset 2. Only one of these plants (*Deinbollia* sp.*)* was visited by birds (Table S1). By supplementing dataset

2 with dataset 1, we gained detailed information on the bird visitation frequencies (i.e. number of individuals per hour of observation) for 102 plant species in total. These visitation frequencies were assessed separately for each study site, as well as for the dry and wet season.

Statistical analysis

To visualise the distribution of plant species within the dimensional space defined by floral traits, we used Factor Analysis of Mixed Data (FAMD), a principal component method dedicated to analysing datasets containing both quantitative and qualitative variables (Pagès, 2004). The size of the centroids was set to reflect the frequency of bird-plant interactions. For this purpose, the frequencies were averaged for those plant species that occurred at more sites, or during both seasons.

Following the concept model (Fig. 1), a chi-squared contingency test was applied to assess whether the distribution of interactions (bird-plant vs. insect-plant) was different from the expected random distribution. This included three separate tests analysing: 1) if the number of common interactions of plants shared by birds and insects (zones II and III in Fig. 1) differed from the number of specific interactions (zones I and IV, Fig. 1); 2) if the plants within the bird pollination syndrome were visited by birds (i.e. occurred in zone I and II, Fig. 1) at a higher rate than expected (i.e. equally distributed across all (I-IV) zones); 3) if the plants within the bird pollination syndrome were visited by birds at a higher rate than by insects (number of interactions within zone I versus zone II, Fig. 1). To analyse whether birds fed mainly on ornithophilous plants (Fig. 1c), we applied the Wilcoxon test and compared the mean ranks of visitation frequency between the ornithophilous and non-ornithophilous plants.

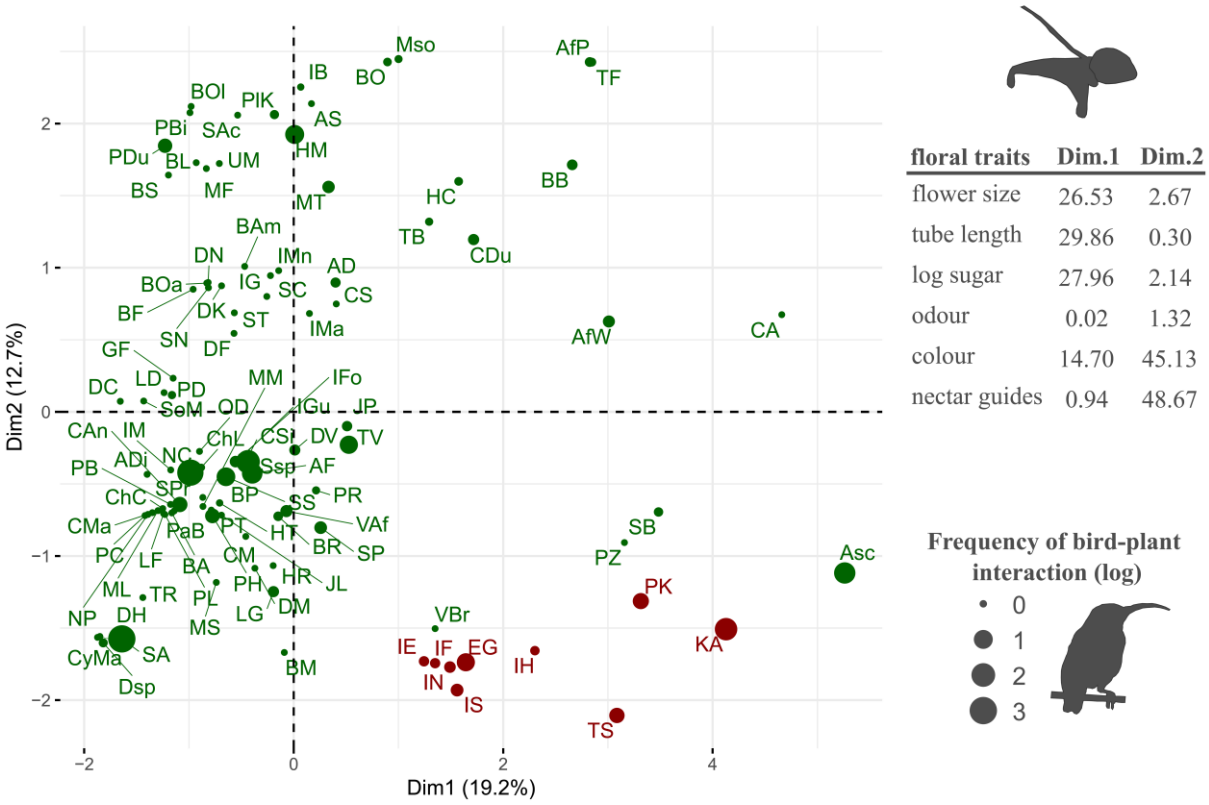
We used a Compound Poisson Generalised Linear Mixed Model (CPGLMM; Zhang, 2013) to assess the effect of floral resource quantity and individual floral traits (fixed effects) on bird visitation frequencies. This approach enabled us to handle zero inflation of our data.

To account for the potential spatiotemporal patterns and abundance of flowering plants in our dataset, we set the site, season and abundance of flowering plants per hectare as random effects. For the plants which were relatively common at the study site but did not flower on the transects, we arbitrarily set their abundance as 1 individual per 2 hectares. For individual floral traits, we analysed the conditional effect, meaning that the effect of each floral trait was tested within a full model (six traits) against a model containing all floral traits minus the one being tested (five traits). Additionally, we computed the marginal R^2 (explained variation) from CPLGLMM models following Nakagawa *et al.* (2017). This, together with goodness-of-fit (AIC), allowed us to compare the relative effect of resource quantity and the set of floral traits in relation to the bird visitation frequencies.

Results

Bird visitors were observed feeding on 48 plant species. Nine of these plant species fit the bird pollination syndrome (Fig. 2a-i). Overall, the studied plant species expressed a wide diversity of flowers with substantial differences in the composition of individual floral traits (Fig. 3).

Figure 2. Flowers of the nine ornithophilous (a-i, left red block) and nine most visited non-ornithophilous (j-r, right green block) plant species. Both ornithophilous and non-ornithophilous plants are ordered by decreasing frequency of bird-plant interaction. a: *Kigelia africana*, b: *Englerina gabonensis*, c: *Phragmathera kamerunensis*, d: *Thonningia sanguinea*, e: *Impatiens sakeriana*, f: *Impatiens niamniamensis*, g: *Impatiens etindensis*, h: *Impatiens frithii*, i: *Impatiens hians*, j: *Shefflera abyssinica*, k: *Nuxia congesta*, l: *Syzygium sp*, m: *Anthocleista scandens*, n: *Anthonotha fragrans*, o: *Hugonia micans*, p: *Syzygium staudtii*, q: *Tabernemontana ventricosa*, r: *Clausena anisata*. Photographs were provided by authors.



floral traits	Dim.1	Dim.2
flower size	26.53	2.67
tube length	29.86	0.30
log sugar	27.96	2.14
odour	0.02	1.32
colour	14.70	45.13
nectar guides	0.94	48.67

Figure 3. Ordination diagram (FAMD) showing the distribution of 102 plant species (red = ornithophilous plant species, green = other plant species) within the morpho-space defined by the composition of six floral traits: colour, odour, presence of nectar guides, size, tube length and log sugar content per flower. Point size designates the frequency of bird visitors (on a logarithmic scale). The first (dim1) and second (dim2) axes explained 19.2% and 12.7% of the variation, respectively. Contributions (%) of individual explanatory variables to the explained variation by dim1 and dim2 are shown next to the diagram. Species codes: AD - *Acanthopale decempedalis*, Adi - *Anchomanes difformis*, AF - *Anthonotha fragrans*, AfP - *Aframomum sp purple*, AfW - *Aframomum sp white*, AS - *Acanthonema strigosum*, Asc - *Anthocleista scandens*, BA - *Basella alba*, BAm - *Begonia ampla*, BB - *Berlinia bracteosa*, BF - *Begonia fusialata*, BL - *Begonia letouzeyi*, BM - *Begonia mannii*, BO - *Brillantaisia owariensis*, BOa - *Begonia oxyanthera*, BOl - *Begonia oxyloba*, BP - *Begonia poculifera*, BR - *Bertiera racemosa*, BS - *Begonia scutifolia*, CA - *Calochone acuminata*, CAn - *Clausena anisata*, Cdu - *Costus dubius*, CM - *Crassocephalum montuosum*, CMa - *Culcasia mannii*, CS - *Calanthe sylvatica*, Csi - *Clerodendrum silvanum*, CyMa - *Cyphostemma mannii*, DC - *Dioscoreophyllum cumminsii*, DF - *Distephanus biafrae*, DH - *Discocloaxylon hexandrum*, DK - *Disperis kamerunensis*, DM - *Dorstenia*

mannii, DN - *Disperis nitida*, Dsp - *Deinbollia sp 1*, DV - *Dicranolepis vestita*, EG - *Englerina gabonensis*, GF - *Gomphia flava*, HC - *Heinsia crinita*, HM - *Hugonia micans*, HR - *Hylodesmum repandum*, HT - *Hypoestes triflora*, ChC - *Chlorophytum comosum*, ChL - *Chassalia laikomensis*, IB - *Impatiens burtonii*, IE - *Impatiens etindensis*, IF - *Impatiens frithii*, IFo - *Ixora foliosa*, IG - *Isoglossa glandulifera*, IGu - *Ixora guineensis*, IH - *Impatiens hians*, IM - *Ilex mitis*, IMa - *Impatiens macroptera*, Imn - *Impatiens mannii*, IN - *Impatiens niamniamensis*, IS - *Impatiens sakeriana*, JL - *Justicia laxa*, JP - *Jasminum preussii*, KA - *Kigelia africana*, LD - *Liparis deistelii*, LF - *Laccodiscus ferrugineus*, LG - *Leea guineensis*, MF - *Momordica foetida*, ML - *Maesa lanceolata*, MM - *Marantochloa monophylla*, MS - *Melanthera scandens*, Mso - *Mimulopsis solmsii*, MT - *Mussaenda tenuiflora*, NC - *Nuxia congesta*, NP - *Nephthytis poissonii*, OD - *Oncoba dentata*, PaB - *Psychotria bifaria*, PB - *Palisota barteri*, PBi - *Polystachia bicalcarata*, PC - *Piper capense*, PD - *Plectranthus decurrens*, PDu - *Psydrax dunlapii*, PH - *Psychotria hypsophila*, PK - *Phragmanthera kamerunensis*, PL - *Psychotria leptophylla*, PlK - *Plectranthus kamerunensis*, PR - *Pavetta rigida*, PT - *Psychotria thonneri*, PZ - *Pararistolochia zenkeri*, SA - *Schefflera abyssinica*, SAc - *Stachys aculeolata*, SB - *Sherbournia bignoniiflora*, SC - *Sabicea calycina*, SN - *Solanum nigrum*, SoM - *Solanecio mannii*, SP - *Sabicea pilosa*, SPr - *Spermacoce princeae*, SS - *Syzygium staudtii*, Ssp - *Syzygium sp* , ST - *Solanum terminale subsp inconstans*, TB - *Tabernaemontana brachyantha*, TF - *Thunbergia fasciculata*, TR - *Trichilia rubescens*, TS - *Thonningia sanguinea*, TV - *Tabernemontana ventricosa*, UM - *Utricularia mannii*, VAf - *Voacanga africana*, VBr - *Voacanga bracteata*

The plants were evenly distributed in the bird-plant x insect-plant interaction space (Chi-square test, $df_{44,44} = 1$, $\chi^2 = 0$, $p = 1.000$), whilst half (21) out of the 42 plant species visited by birds had a larger proportion of bird to insect rank visitation (Fig. 4). The other half of the plant species were similarly important for both bird and insect visitors (Fig. 4). The ornithophilous plants occurred in the bird-favoured area (i.e. zones I and II in Figure 1) more often than expected by chance (Chi-square test, $df_{9,0} = 1$, $\chi^2 = 9.00$, $p = 0.003$) (Fig. 4).They hosted a only marginally larger proportion of bird to insect rank visitation, i.e. visits occurred

within the bird-plant interaction zone (zone I in Figure 1) at a higher rate than expected by chance (Chi-square test, $df_{7,2} = 1$, $\chi^2 = 2.77$, $p = 0.096$) (Fig. 4). However, when compared with the other plants visited by birds (e.g. Fig. 2j-r), the ornithophilous plants did not show higher frequencies of bird visits (Wilcoxon test, $df_{9,39} = 1$, $W = 120$, $p = 0.146$). Altogether, plants visited by birds did not occupy a unique portion of the trait space, meaning that we did not observe distinct separation between visited and non-visited plants in terms of the composition of floral traits (Fig. 3).

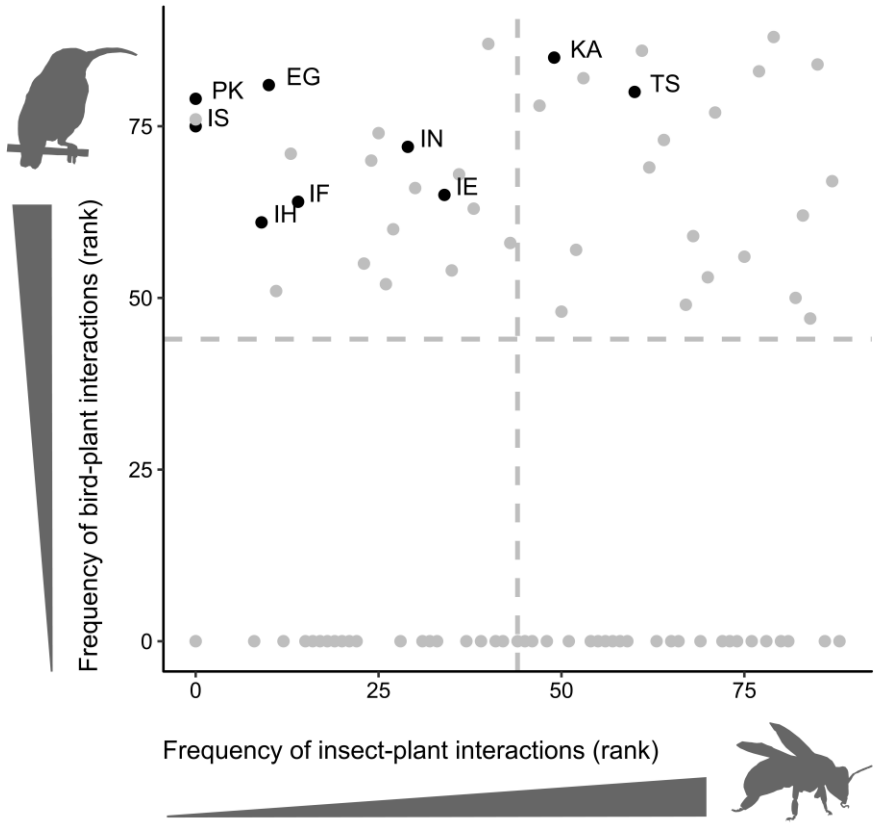


Figure 4. Relationship between ranked bird and insect frequencies on 88 different plant species. Plant species forming a bird pollination syndrome (EG = *Englerina gabonensis*, IE = *Impatiens etindensis*, IF = *Impatiens frithii*, IH = *Impatiens hians*, IN = *Impatiens niamniamensis*, KA = *Kigelia africana*, PK = *Phragmanthera kamerunensis*, TS = *Thonningia sanguinea*) are visualised in black, the rest in grey.

Although birds did not seem to prefer flowers of a specific phenotype, the floral traits still explained a relatively high proportion of variability in the frequency of bird-plant interactions ($R^2 = 0.2132$). A comparable proportion was explained by the quantity of nectar resource offered by an individual plant species ($R^2 = 0.2076$). However, the resource quantity itself explained more variability in the visitation frequencies than any other floral trait. Moreover, in terms of relative quality (AIC), the resource quantity was a better predictor, with a positive effect on the visitation frequency (Table 1, Fig. 5). Among the individual floral traits: odour, sugar content, tube length and presence/absence of nectar guides were significant predictors of visitation frequency as well (ordered by decreasing amount of explained variability; Table 1). On the contrary, visitation frequency was not affected by the floral colour and size (Table 1). Bird visitation frequency tended to be higher on flowers: that exuded odour, with increased sugar content, with longer tube length, and with the absence of nectar guides (Table 1).

Table 1. Influence of individual floral traits on bird visitor frequency. CPGLMM, n = 184, elevation and season were set as random effects. Models are ordered by marginal R^2, computed with the function provided by Nakagawa (2017). Colour was tested as a two-level factor: orange-red vs. other colours. We indicate the direction of significant relationships with bird frequency, where ++ means positive for continuous variables, whereas the factor with the highest value of bird frequency is shown for qualitative variables.

	model	R^2 marginal	AIC	Chisq	p	direction of relationship
~zero model		0.0000	263.38			
~resource quantity		0.2076	159.03	106.34	**<0.001**	++
~floral traits		0.2132	211.35	64.03	**<0.001**	
Contribution of individual floral traits	odour	0.0957	-	22.66	**<0.001**	exude odour ++
	sugar content	0.0333	-	23.78	**<0.001**	++
	tube length	0.0284	-	4.70	**0.030**	++
	nectar guides	0.0146	-	4.61	**0.031**	absent ++
	colour	0.0032	-	0.62	0.430	n.s.
	flower size	0.0014	-	0.29	0.590	n.s.

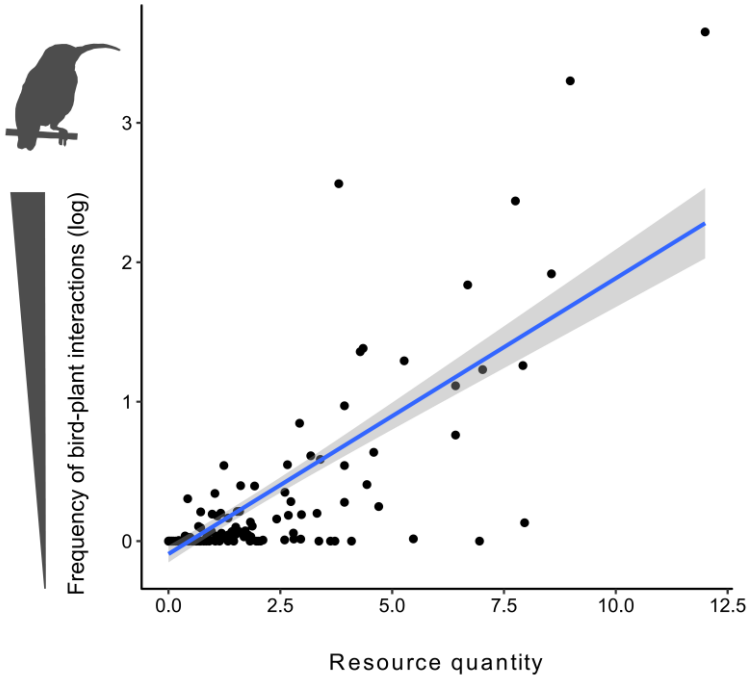


Figure 5. Relationship between bird visitation frequency and resource quantity (nectar sugar content per average plant individual). Shaded areas represent the standard error. Logarithmic transformation was used for both variables.

Discussion

Plants evenly occupy the insect-plant x bird-plant interaction space

We documented a regular distribution of plants in the four zones of niche space delimited by bird-plant and insect-plant interaction axes. Based on the conceptual model, an equal proportion of plants tended to be specialised either towards birds or insects (i.e. plants in zones I and IV in the conceptual model) and the same proportion consisted of generalised plants, where the ranked interaction frequencies of both visitor groups overlapped (zones II and III). This pattern therefore partly showed the high diversification of pollination niches (Johnson, 2010), and partly supported the ideas of Waser *et al.* (1996) and Ollerton *et al.* (2009) who underlined that a relatively large proportion of flowers do not host a specific pollinator. Although we observed similar numbers of plants in the four zones of our conceptual framework, the regularity of the plant distribution in the bird non-preferred zones (i.e. zones III and IV in Figure 1) was somewhat disrupted by the fact that these plants were not visited by birds at all. We assume that there is a certain threshold related to plant profitability, which makes these plants unattractive for birds. Conversely, we found only three plant species exclusively visited by birds with no records of insect visitors. We propose two primary explanations for the fact why ornithophilous plants are still at least somewhat attractive for insects (see also Wilson *et al.*, 2004): 1) plants adapted to birds usually produce a lot of nectar, which is, despite other anti-insect adaptations, acts as a strong attractant, 2) flowers also offer other insect rewards such as pollen.

Validity of the bird pollination syndrome

Our results showed that the set of floral traits described representing the bird pollination syndrome (Faegri & van der Pijl, 1979; Fenster *et al.*, 2004; Cronk & Ojeda, 2008) were indeed an indicator of plant specialisation towards bird pollination. The ornithophilous plant species

were visited by birds at higher frequencies than by chance, and most of them were ranked higher by birds than by insects. Only two species, *Kigelia africana* and *Thonningia sanguinea*, which according to their floral traits were affiliated to the bird pollination syndrome, were not preferred by birds exclusively, but also by insects. This is in agreement with studies from other regions showing that the interactions of these two plant species were more complex. When considering additional floral traits, these species did not perfectly fit the bird pollination syndrome (e.g. Quintero *et al.*, 2017; Namah, Midgley, & Kruger, 2019). While Namah *et al.* (2019) described common interactions of *Kigelia africana* with birds and insects, reflecting our findings, other studies demonstrated *K. africana* was primarily pollinated by fruit bats (Baker, 1961; Ayensu, 1974). The pollination system of *Thonningia sanguinea* is similarly ambivalent, as both flies (Goto, Yamakoshi, & Matsuzawa, 2012) and birds (Quintero *et al.*, 2017) were documented as its main pollinators. The other ornithophilous plants, which were affiliated only to the bird-plant interaction zone, were shown in other studies to be bird pollinated (Janeček *et al.*, 2011; Janeček, Bartoš, & Njabo, 2015; Bartoš & Janeček, 2014).

Regardless of any irregularities mentioned above, our study supports the validity of the bird pollination syndrome hypothesis. Hence, it is another example demonstrating that reddish tubular flowers with copious nectar reward are not a form of adaptation exclusive to the most studied nectar specialist – New World's hummingbirds (e.g. Dziedzioch, Stevens, & Gottsberger, 2003; Muchhala, 2006), but such traits represent a convergent evolutionary shift to other specialised groups of birds such as sunbirds (Rodríguez-Gironés & Santamaría, 2004; Fleming & Muchhala, 2008). Nevertheless, the representation of ornithophilous plant species in Afrotropical rainforests appears to be much lower compared to the Neotropics. Stiles (1985) documented 50 plant species pollinated primarily by hummingbirds in Costa Rican submontane forest. In a montane forest in the Andes, Dziedzioch *et al.* (2003) observed hummingbirds feeding on 67 plant species of which half had red tubular flowers. In the Atlantic

Forest, including lowland and highland sites, Buzato *et al.* (2000) documented 86 hummingbird-pollinated plant species. Whereas, in our study, out of the 42 studied plants, only nine met the characteristics of ornithophilous flowers.

Effectiveness of birds as pollinators

Considering the relationship from a plant perspective, it is important to acknowledge that the interaction frequency is not the only parameter determining pollinator effectiveness. This can in fact be much higher in specialised plants, because of various floral adaptations (e.g. Thomson *et al.*, 2000; Hargreaves, Johnson, & Nol, 2004). Birds have been shown to be highly effective pollinators during just a single visit of the ornithophilous plant *Impatiens sakeriana* (Janeček *et al.*, 2011). For example, the effectiveness can be increased by different mechanisms allowing specific and precise pollen placement on parts of the bird body (Bartoš & Janeček, 2014). Such placement prevents heterospecific pollen deposition on stigmas (Morales & Traveset, 2008). Ornithophilous plants do not necessarily need to receive many visits, but rather simply a few effective ones in order to reproduce.

Birds can likely also act as effective pollinators on numerous non-ornithophilous plants. For example, even if individual flowers were very small (e.g. *Nuxia congesta* or *Syzygium sp.*, Fig. 2k,l), birds perching directly on the inflorescences will likely transfer pollen to their feet or other body parts as shown in a recent study of a montane system in Nigeria (Nsor, Godsoe, & Chapman, 2019).

On the other hand, we are aware that not all visitors attracted to flowers act as pollinators. We witnessed how some birds participated in nectar robbing, i.e. removing nectar without contacting the anthers and/or stigma (reviewed in Irwin *et al.*, 2010). Although we were not able to quantify the proportion of nectar robbing for individual plant species, we did not observe exclusive nectar stealing for any plant species.

Therefore, in many cases, the simple visitation rate likely does not reflect the pollination effectiveness, measured for example as germinated pollen load per visit (Rocca & Sazima, 2013). Here we focused primarily on the visitor perspective, i.e. what defines the attractivity of a plant and the floral choice. At this level, the effectiveness of pollination is not so important, although it would be extremely valuable to unravel the relationship between the visitation rate and the pollination effectiveness in individual plant species.

Birds feed both on ornithophilous and non-ornithophilous plants

Although all studied ornithophilous plants hosted bird visitors, birds also fed frequently on other non-ornithophilous plants. Moreover, their interaction frequencies on these non-ornithophilous plants were not significantly different from the specialised ornithophilous plants. These results agree with other studies showing that phenotypically non-specialised plants are important in the nutrition of sunbirds (Nsor *et al.*, 2019), hummingbirds (Maruyama *et al.*, 2013) and honeyeaters (Castro & Robertson, 1997).

This finding corresponds with the observation that bird adaptations, such as long bill and tongue, enable drinking from specialised tubular flowers, but do not prevent them from also using flowers with more exposed nectar (Janeček *et al.*, 2007, 2012).

It is therefore evident that birds care little about whether they feed from ornithophilous or non-ornithophilous flowers. Nevertheless, we should avoid the interpretation that ornithophilous plants are generally an unimportant source of nectar. They can be a crucial source of nectar in some periods of the year (e.g. wet season) or for some sunbird species (Janeček *et al.*, 2015; Janeček *et al.* unpublished data)

Resource quantity as a best predictor of floral choice

Our findings support those of other studies which suggest floral choice is governed primarily by profitability – the amount and availability of nectar reward (Pleasants & Waser, 1985; Collins & Newland, 1986; Schmid *et al.*, 2016).

Although nectar production per plant appears to be the most important trait for birds, additional flower traits are also at play. Some plant species may produce a large quantity of nectar per plant individual, which would potentially attract bird visitors, but this resource may be inaccessible (Grant & Temeles, 1992). For instance, nectar containing floral tubes are likely too narrow for a bird's beak in few of the sampled species (*e.g. Heinsia crinita* or *Impatiens mannii*). In other cases, plants may produce a large quantity of nectar, but through super abundant small stellate flowers with a very low amount of nectar per flower (e.g. *Ilex mitis*), thus making the handling of flowers unprofitable for birds. On the contrary, some plant traits that have evolved to attract other pollinator groups did not deter birds. We found that the frequency of birds tended to be higher on flowers that exert odour, despite the fact that this trait plays a prominent role in the location of flowers by insects, especially in nocturnal bat and moth-pollinated flowers (Faegri & van der Pijl, 1979). The colour of flowers did not influence the frequency of feeding birds, suggesting that bird floral choice is mainly based on previous experience. During which birds tested how much nectar the plant offers and whether it is accessible, rather than on *a priori* visual cues of the flowers (Wolf & Hainsworth, 1983).

Furthermore, we are aware that the floral choice will be additionally affected by competition and resource partitioning. This may prevent birds from visiting the seemingly richest nectar resource and may account for some of the unexplained variation in bird visitation rates. Here we assume there is no difference in flowering plant preference between all visitors. Nevertheless, resource partitioning is generally common within nectarivorous bird communities, with smaller species often being forced to forage on less productive plants (Gill

& Wolf, 1975; Ford & Paton, 1982; Janeček *et al.*, 2012). Additionally, intraspecific differences in foraging patterns have also been described (Dupont *et al.*, 2014; Maruyama, Justino, & Oliveira, 2016).

Conclusions

This analysis of plant-pollinator interactions on Mt. Cameroon revealed that despite specialised ornithophilous plants receiving a higher proportion of legitimate visitors, birds fed frequently on other unspecialised plants. From a birds' perspective, the attractiveness of each plant is defined primarily by the nectar resource availability and it seems to be a better predictor of bird visitation compared to the traditional floral traits related to the bird pollination syndrome.

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Supplementary information

Supplementary Table 1. Dataset used, including visitor observations and floral traits for studied plant species per season and elevation.

site	season	species	code	life form*	dataset**	observed hours	number of flowering induals/ha	mean number of flowers	bird visitor frequency	bird pollination syndrome	odour	colour	colours two factors	nectar guides	mean size [cm]	tube length [cm]	sugar content [mg] per flower	sugar content [mg] per average individual
650	dry	Acanthonema strigosum	AS	HSL	1	227.0167	28.33333	1.852941	0	n	Weak-No	Purple	o	Present	1.184385	1.682846	0.060979	0.112991
650	wet	Acanthonema strigosum	AS	HSL	1	95.85	94.16667	1.212389	0	n	Weak-No	Purple	o	Present	1.184385	1.682846	0.060979	0.07393
650	dry	Aframomum sp purple	AfP	HSL	1	218.65	14.16667	1.294118	0.712707	n	Weak-No	Purple	o	Present	5.124313	4.5085	1.054721	1.364933
650	wet	Aframomum sp purple	AfP	HSL	1	40.5	0.833333	1	0	n	Weak-No	Purple	o	Present	5.124313	4.5085	1.054721	1.054721
650	dry	Anchomanes difformis	ADi	HSL	2	111.7167	0.833333	250	0	n	Mod-strong	White	o	Absent	0.245778	0	0.003995	0.99881
650	dry	Anthonotha fragrans	AF	T	1	74.3	1.666667	6000	9.668013	n	Mod-strong	White	o	Absent	1.366	0.75	0.870234	5221.401
650	wet	Begonia ampla	BAm	HSL	2	80.65	6.666667	1.375	0	n	Weak-No	White	o	Present	2.622625	0	0.000306	0.000421
650	dry	Begonia letouzeyi	BL	HSL	2	74.15833	6.666667	2.375	0	n	Weak-No	Yellow	o	Present	2.0845	0	0.004188	0.009947
650	dry	Begonia mannii	BM	HSL	2	168.55	8.333333	3.7	0	n	Weak-No	Red	redorange	Absent	1.5245	0	0.004596	0.017006
650	wet	Begonia mannii	BM	HSL	2	168.55	31.66667	7.552632	0	n	Weak-No	Red	redorange	Absent	1.5245	0	0.004596	0.034713
650	wet	Begonia scutifolia	BS	HSL	2	48.525	25	1.666667	0	n	Weak-No	Yellow	o	Present	1.169429	0	0.008969	0.014949
650	dry	Berlinia bracteosa	BB	T	1	64	4.166667	1400	0.585938	n	Mod-strong	White	o	Present	9.12075	1.459	2.046442	2865.019
650	wet	Bertiera racemosa	BR	T	1	70.61667	0.5	5	0.035402	n	Weak-No	White	o	Absent	0.655333	2.072667	0.628289	3.141446
650	wet	Clerodendrum silvanum	CSi	HSL	1	118.1333	30.83333	54.86486	0.261005	n	Mod-strong	White	o	Absent	1.3895	1.2433	0.226295	12.41567
650	dry	Culcasia mannii	CMa	HSL	2	49.575	2.5	12.66667	0	n	Weak-No	White	o	Absent	0.3088	0	0.006292	0.079703

650	dry	Deinbollia sp 1	Dsp	HSL	2	89.85833	3.333333	17	0.148382	n	Weak-No	Green	o	Absent	0.2886	0	0.164493	2.796379
650	wet	Dioscoreophyllum cumminsii	DC	HSL	2	48.275	0.833333	30	0	n	Weak-No	Yellow	o	Absent	0.3216	0	0.002707	0.08121
650	dry	Dorstenia mannii	DM	HSL	2	36.78333	5	113.3333	0	n	Weak-No	Green	o	Absent	5.5531	0	0.000326	0.036904
650	dry	Gomphia flava	GF	HSL	2	115.3417	0.833333	30	0	n	Weak-No	Yellow	o	Absent	2.0406	0	0.004195	0.125844
650	dry	Heinsia crinita	HC	T	1	32	2.5	24.33333	0.078125	n	Mod-strong	White	o	Present	6.013286	2.524429	0.181229	4.409897
650	dry	Hugonia micans	HM	HSL	1	48.03333	0.5	104.49	1.321999	n	Mod-strong	Yellow	o	Present	3.191	0	1.849996	193.3061
650	dry	Chlorophytum comosum	ChC	HSL	2	109.825	1.666667	2	0	n	Weak-No	White	o	Absent	0.6308	0	0.014657	0.029314
650	wet	Chlorophytum comosum	ChC	HSL	2	109.825	0.833333	1	0	n	Weak-No	White	o	Absent	0.6308	0	0.014657	0.014657
650	wet	Impatiens hians	IH	HSL	1	110.05	4.166667	2.6	0.227169	y	Weak-No	Red	redorange	Absent	2.951389	3.573778	1.613125	4.194126
650	wet	Impatiens macroptera	IMa	HSL	1	134.6333	80.83333	1.175258	0	n	Weak-No	Pink	o	Absent	2.6141	1.4807	0.754631	0.886886
650	dry	Impatiens niamniamensis	IN	HSL	1	237.3	5.833333	2	0.614553	y	Weak-No	Red	redorange	Absent	2.0146	2.1059	1.74866	3.49732
650	wet	Impatiens niamniamensis	IN	HSL	1	124.7	19.16667	2.173913	1.38332	y	Weak-No	Red	redorange	Absent	2.0146	2.1059	1.74866	3.801435
650	dry	Ixora guineensis	IGu	T	1	241.8667	66.66667	58.15	0	n	Mod-strong	White	o	Absent	1.4318	1.3668	0.047823	2.78091
650	wet	Justicia laxa	JL	HSL	1	105.1	25.83333	2.419355	0	n	Weak-No	White	o	Absent	0.8377	0.6152	0.549468	1.329358
650	dry	Laccodiscus ferrugineus	LF	HSL	2	61.86667	3.333333	26.75	0	n	Weak-No	White	o	Absent	0.4436	0	0.123382	3.300457
650	wet	Leea guineensis	LG	HSL	1	55.55	2.5	3.333333	0.450045	n	Weak-No	Orange	redorange	Absent	0.575	0.3765	0.835819	2.786064
650	wet	Marantochloa monophylla	MM	HSL	1	41.2	6.666667	1.625	0	n	Weak-No	White	o	Absent	0.8494	0.6924	0.103367	0.167972
650	dry	Melanthera scandens	MS	HSL	2	197.3167	0.833333	10	0	n	Weak-No	Orange	redorange	Absent	0.168	0.2417	0.002905	0.029048
650	wet	Melanthera scandens	MS	HSL	2	197.3167	0.833333	100	0	n	Weak-No	Orange	redorange	Absent	0.168	0.2417	0.002905	0.290482
650	dry	Nephthytis poissonii	NP	HSL	2	73.275	5	25.83333	0	n	Weak-No	White	o	Absent	0.17525	0	0.002431	0.062795
650	wet	Nephthytis poissonii	NP	HSL	2	73.275	0.833333	1	0	n	Weak-No	White	o	Absent	0.17525	0	0.002431	0.002431
650	dry	Oncoba dentata	OD	HSL	2	74.80833	1.666667	8.5	0	n	Mod-strong	White	o	Absent	1.952	0	0.004662	0.039623
650	dry	Palisota barteri	PB	HSL	2	179.5417	0.833333	90	0	n	Weak-No	White	o	Absent	0.912286	0	0.000891	0.080214
650	wet	Palisota barteri	PB	HSL	2	179.5417	9.166667	13.90909	0	n	Weak-No	White	o	Absent	0.912286	0	0.000891	0.012397
650	dry	Pararistolochia zenkeri	PZ	HSL	2	35.99167	2.5	3.333333	0	n	Weak-No	Brown	o	Absent	5.1534	5.1604	0.030093	0.100309
650	wet	Phragmanthera kamerunensis	PK	HSL	1	77.2	0.833333	140	0.949914	y	Weak-No	Orange	redorange	Absent	0.925	7.46	4.3677	611.478
650	wet	Plectranthus decurrens	PD	HSL	1	108.9667	8.333333	3.2	0	n	Weak-No	Yellow	o	Absent	0.8742	0.6996	0.105834	0.338669
650	dry	Psychotria bifaria	PaB	HSL	2	49.25833	4.166667	3.2	0	n	Weak-No	White	o	Absent	0.4438	0.3884	0.037298	0.119355
650	dry	Psychotria leptophylla	PL	HSL	2	97.91667	4.166667	1.8	0	n	Weak-No	White	o	Absent	0.2982	0.1862	0.093163	0.167693

650	wet	Psychotria leptophylla	PL	HSL	2	97.91667	15	19.83333	0	n	Weak-No	White	o	Absent	0.2982	0.1862	0.093163	1.847723
650	wet	Psychotria thonneri	PT	HSL	1	55.81667	32.5	2.076923	0	n	Weak-No	White	o	Absent	0.5028	1.1736	0.094604	0.196486
650	dry	Sabicea calycina	SC	HSL	1	226.8333	6.666667	4.875	0	n	Weak-No	White	o	Present	0.6708	1.599333	0.304224	1.483093
650	wet	Sabicea calycina	SC	HSL	2	48.90833	0.833333	7	0	n	Weak-No	White	o	Present	0.6708	1.599333	0.304224	2.129569
650	dry	Sabicea pilosa	SP	HSL	1	229.3333	11.66667	2.428571	5.697674	n	Weak-No	White	o	Absent	0.6761	2.3245	1.653337	4.015247
650	dry	Solanum terminale subsp inconstans	ST	HSL	2	90.60833	0.833333	1	0	n	Weak-No	Purple	o	Absent	1.7255	0	0.000893	0.000893
650	wet	Solanum terminale subsp inconstans	ST	HSL	2	90.60833	20	2.291667	0	n	Weak-No	Purple	o	Absent	1.7255	0	0.000893	0.002046
650	dry	Tabernaemontana brachyantha	TB	T	1	62	11.66667	125.3571	0.188172	n	Mod-strong	White	o	Present	4.827571	1.259571	1.894523	237.4919
650	dry	Voacanga bracteata	VBr	HSL	1	208.2667	3.333333	2.75	0	n	Weak-No	Brown	o	Absent	1.404	1.4454	2.338865	6.43188
1100	wet	Acanthonema strigosum	AS	HSL	1	90.25	11.66667	1.214286	0	n	Weak-No	Purple	o	Present	1.184385	1.682846	0.060979	0.074046
1100	dry	Aframomum sp purple	AfP	HSL	1	217.6333	9.166667	1.363636	0.126359	n	Weak-No	Purple	o	Present	5.124313	4.5085	1.054721	1.438256
1100	wet	Anchomanes difformis	ADi	HSL	2	111.7167	1.666667	300	0	n	Mod-strong	White	o	Absent	0.245778	0	0.003995	1.198571
1100	dry	Anthonotha fragrans	AF	T	1	44	5.833333	3171.429	14.71591	n	Mod-strong	White	o	Absent	1.366	0.75	0.870234	2759.883
1100	wet	Basella alba	BA	HSL	2	36.88333	5	13.83333	0	n	Weak-No	White	o	Absent	0.2262	0.5388	0.008372	0.11581
1100	dry	Begonia ampla	BAm	HSL	2	80.65	1.666667	1.5	0	n	Weak-No	White	o	Present	2.622625	0	0.000306	0.000459
1100	wet	Begonia ampla	BAm	HSL	2	80.65	13.33333	1.3125	0	n	Weak-No	White	o	Present	2.622625	0	0.000306	0.000402
1100	wet	Begonia fusialata	BF	HSL	2	56.94167	4.166667	1.6	0	n	Weak-No	White	o	Present	0.9329	0	0.004919	0.007871
1100	dry	Begonia letouzeyi	BL	HSL	2	74.15833	0.833333	4	0	n	Weak-No	Yellow	o	Present	2.0845	0	0.004188	0.016754
1100	dry	Begonia mannii	BM	HSL	2	168.55	2.5	80	0	n	Weak-No	Red	redorange	Absent	1.5245	0	0.004596	0.367692
1100	wet	Begonia mannii	BM	HSL	2	168.55	19.16667	7.956522	0	n	Weak-No	Red	redorange	Absent	1.5245	0	0.004596	0.036569
1100	wet	Begonia oxyloba	BOl	HSL	2	146.5167	17.5	4.190476	0	n	Weak-No	Pink	o	Present	1.1908	0	0.000967	0.004053
1100	wet	Begonia scutifolia	BS	HSL	2	48.525	2.5	1.333333	0	n	Weak-No	Yellow	o	Present	1.169429	0	0.008969	0.011959
1100	dry	Brillantaisia owariensis	BO	HSL	1	224.45	1.666667	4.5	0	n	Mod-strong	Purple	o	Present	2.9276	1.0362	1.361855	6.128348
1100	wet	Calanthe sylvatica	CS	HSL	1	112.6833	2.5	5.333333	0	n	Weak-No	Purple	o	Absent	2.10577	2.2196	0.005723	0.030524
1100	dry	Calochone acuminata	CA	HSL	1	108.3833	0.5	6.73	0	n	Weak-No	Orange	redorange	Present	7.74	4.2888	8.742743	58.83866
1100	dry	Costus dubius	CDu	HSL	1	205.6333	0.833333	10	0.012158	n	Mod-strong	White	o	Present	3.1411	3.674	1.822662	18.22662
1100	wet	Costus dubius	CDu	HSL	1	117.75	0.833333	1	0.339703	n	Mod-strong	White	o	Present	3.1411	3.674	1.822662	1.822662
1100	dry	Crassocephalum montuosum	CM	HSL	2	157.4667	1.666667	225	0	n	Mod-strong	Orange	redorange	Absent	0.6139	0.7089	0.00259	0.582766
1100	wet	Crassocephalum montuosum	CM	HSL	2	157.4667	9.166667	66.45455	0	n	Mod-strong	Orange	redorange	Absent	0.6139	0.7089	0.00259	0.172122

1100	dry	Deinbollia sp 1	Dsp	HSL	2	89.85833	0.833333	30	0.037095	n	Weak-No	Green	o	Absent	0.2886	0	0.164493	4.934787
1100	dry	Discocloaxylon hexandrum	DH	T	2	60.525	0.833333	1	0	n	Weak-No	Green	o	Absent	0.492	0	0.00449	0.00449
1100	dry	Gomphia flava	GF	HSL	2	115.3417	3.333333	65.75	0	n	Weak-No	Yellow	o	Absent	2.0406	0	0.004195	0.275808
1100	wet	Gomphia flava	GF	HSL	2	115.3417	1.666667	1	0	n	Weak-No	Yellow	o	Absent	2.0406	0	0.004195	0.004195
1100	wet	Impatiens etindensis	IE	HSL	1	91.18333	0.833333	2	0.027417	y	Weak-No	Red	redorange	Absent	2.027	1.848143	1.151554	2.303107
1100	wet	Impatiens frithii	IF	HSL	1	153.3167	6.666667	1	0.739211	y	Weak-No	Red	redorange	Absent	1.6173	2.5836	0.957266	0.957266
1100	wet	Impatiens hians	IH	HSL	1	124.3167	0.833333	1	0.06033	y	Weak-No	Red	redorange	Absent	2.951389	3.573778	1.613125	1.613125
1100	wet	Impatiens mannii	IMn	HSL	1	44.91667	13.33333	1.375	0	n	Weak-No	White	o	Present	2.27125	1.0241	0.052178	0.071745
1100	dry	Impatiens niamniamensis	IN	HSL	1	240.35	0.833333	2	0.041606	y	Weak-No	Red	redorange	Absent	2.0146	2.1059	1.74866	3.49732
1100	wet	Impatiens niamniamensis	IN	HSL	1	121.8333	12.5	1.266667	2.770178	y	Weak-No	Red	redorange	Absent	2.0146	2.1059	1.74866	2.214969
1100	dry	Kigelia africana	KA	T	1	60.18333	8.333333	4	99.83384	y	Weak-No	Red	redorange	Absent	7.4974	2.4106	11.00024	44.00097
1100	dry	Melanthera scandens	MS	HSL	2	197.3167	30	138.3333	0	n	Weak-No	Orange	redorange	Absent	0.168	0.2417	0.002905	0.401833
1100	wet	Melanthera scandens	MS	HSL	2	197.3167	9.166667	22	0	n	Weak-No	Orange	redorange	Absent	0.168	0.2417	0.002905	0.063906
1100	dry	Mussaenda tenuiflora	MT	HSL	1	119.55	0.5	7.1	0	n	Weak-No	Yellow	o	Present	1.130667	2.916444	0.960897	6.82237
1100	wet	Mussaenda tenuiflora	MT	HSL	1	71.6	0.5	3.1375	0.006983	n	Weak-No	Yellow	o	Present	1.130667	2.916444	0.960897	3.014815
1100	dry	Pavetta rigida	PR	HSL	1	132.6333	1.666667	27.5	0.025132	n	Weak-No	White	o	Absent	2.5618	1.6306	0.562756	15.47578
1100	dry	Piper capense	PC	HSL	2	132.8833	3.333333	200	0	n	Weak-No	White	o	Absent	0.0838	0	0.000166	0.03316
1100	wet	Piper capense	PC	HSL	2	132.8833	24.16667	162.4138	0	n	Weak-No	White	o	Absent	0.0838	0	0.000166	0.026928
1100	wet	Plectranthus decurrens	PD	HSL	1	53.38333	3.333333	4.25	0.124883	n	Weak-No	Yellow	o	Absent	0.8742	0.6996	0.105834	0.449795
1100	dry	Psychotria leptophylla	PL	HSL	2	97.91667	0.833333	5	0	n	Weak-No	White	o	Absent	0.2982	0.1862	0.093163	0.465813
1100	wet	Psychotria thonneri	PT	HSL	1	104.25	7.5	1.888889	0	n	Weak-No	White	o	Absent	0.5028	1.1736	0.094604	0.178697
1100	dry	Sabicea pilosa	SP	HSL	1	215.2833	1.666667	1	0.35612	n	Weak-No	White	o	Absent	0.6761	2.3245	1.653337	1.653337
1100	dry	Sherbournia bignoniiflora	SB	HSL	1	134.8667	0.5	1	0.029659	n	Weak-No	White	o	Absent	4.888	4.56525	15.33045	15.33045
1100	dry	Tabernemontana ventricosa	TV	T	1	70	11.66667	16.07143	15.5	n	Mod-strong	White	o	Absent	3.645167	1.22	1.104348	17.74845
1100	wet	Thonningia sanguinea	TS	HSL	1	123.7	5	1	3.597413	y	Weak-No	Red	redorange	Absent	4.062182	0	50.09974	50.09974
1100	wet	Thunbergia fasciculata	TF	HSL	1	107.35	3.333333	1.75	0.124204	n	Weak-No	Purple	o	Present	5.1468	4.5162	1.089872	1.907275
1100	dry	Trichilia rubescens	TR	T	2	107.65	1.666667	100	0	n	Mod-strong	Green	o	Absent	1.005	0.2595	0.363978	36.39778
1100	dry	Voacanga africana	VAf	T	1	64	10	87.91667	2.8125	n	Weak-No	White	o	Absent	1.7764	0.9176	1.239355	108.96
1500	dry	Acanthopale decempedalis	AD	HSL	1	226.2833	9.166667	1.454545	0.121529	n	Weak-No	White	o	Present	1.854714	2.200929	0.476316	0.692823

1500	wet	Acanthopale decempedalis	AD	HSL	1	242.8167	4.166667	2.6	0.034319	n	Weak-No	White	o	Present	1.854714	2.200929	0.476316	1.238421
1500	dry	Aframomum sp purple	AfP	HSL	1	220.4833	3.333333	1	0.332603	n	Weak-No	Purple	o	Present	5.124313	4.5085	1.054721	1.054721
1500	wet	Aframomum sp purple	AfP	HSL	1	90.1	0.5	1	0.116537	n	Weak-No	Purple	o	Present	5.124313	4.5085	1.054721	1.054721
1500	dry	Aframomum sp white	AfW	HSL	1	210.4167	5.833333	1.285714	1.219802	n	Weak-No	White	o	Present	2.996667	4.326667	14.42605	18.54778
1500	wet	Aframomum sp white	AfW	HSL	1	106.85	0.833333	1	0.272968	n	Weak-No	White	o	Present	2.996667	4.326667	14.42605	14.42605
1500	wet	Anthocleista scandens	Asc	HSL	1	64	5	2.833333	26.40625	n	Weak-No	White	o	Present	5.678	3.9928	283.1461	802.2474
1500	wet	Basella alba	BA	HSL	2	36.88333	2.5	23.33333	0	n	Weak-No	White	o	Absent	0.2262	0.5388	0.008372	0.195342
1500	wet	Begonia oxyloba	BOl	HSL	2	146.5167	0.833333	2	0	n	Weak-No	Pink	o	Present	1.1908	0	0.000967	0.001934
1500	wet	Begonia poculifera	BP	HSL	2	171.3167	12.5	2.533333	0	n	Weak-No	White	o	Absent	2.6235	0	0.001256	0.003181
1500	dry	Brillantaisia owariensis	BO	HSL	1	222.9333	2.5	1.666667	0.145783	n	Mod-strong	Purple	o	Present	2.9276	1.0362	1.361855	2.269758
1500	wet	Calanthe sylvatica	CS	HSL	1	80.36667	1.666667	4.5	0	n	Weak-No	Purple	o	Absent	2.10577	2.2196	0.005723	0.025755
1500	wet	Clerodendrum silvanum	CSi	HSL	1	246.0167	1.666667	60	0.33873	n	Mod-strong	White	o	Absent	1.3895	1.2433	0.226295	13.57772
1500	dry	Costus dubius	CDu	HSL	1	170.5833	0.5	2.5	0.038105	n	Mod-strong	White	o	Present	3.1411	3.674	1.822662	4.556654
1500	wet	Costus dubius	CDu	HSL	1	135.1167	0.5	2	0.118416	n	Mod-strong	White	o	Present	3.1411	3.674	1.822662	3.645324
1500	dry	Crassocephalum montuosum	CM	HSL	2	157.4667	16.66667	212.1	0	n	Mod-strong	Orange	redorange	Absent	0.6139	0.7089	0.00259	0.549354
1500	wet	Crassocephalum montuosum	CM	HSL	2	157.4667	0.833333	40	0	n	Mod-strong	Orange	redorange	Absent	0.6139	0.7089	0.00259	0.103603
1500	dry	Dicranolepis vestita	DV	T	1	64	108.3333	37.19231	18.61979	n	Mod-strong	White	o	Absent	2.269833	1.729833	0.275116	10.23219
1500	wet	Disperis nitida	DN	HSL	1	112.0833	1.666667	1.5	0	n	Weak-No	White	o	Present	1.4321	0	0.000133	0.0002
1500	dry	Distephanus biafrae	DF	HSL	2	74.45	3.333333	180	0	n	Weak-No	Purple	o	Absent	0.3572	0.7992	0.15468	27.8424
1500	dry	Englerina gabonensis	EG	HSL	1	64	2.5	500	5.117188	y	Weak-No	Red	redorange	Absent	1.872571	2.945429	1.222826	611.4132
1500	dry	Hylodesmum repandum	HR	HSL	2	99.15833	1.666667	1.5	0	n	Weak-No	Orange	redorange	Absent	1.3473	0.7536	0.002088	0.003131
1500	dry	Hypoestes triflora	HT	HSL	1	235.1	93.33333	4.098214	0	n	Weak-No	White	o	Absent	1.2425	0.6914	0.185328	0.759514
1500	wet	Chassalia laikomensis	ChL	HSL	1	180.0333	4.166667	6.8	0	n	Mod-strong	White	o	Absent	0.832286	0.743143	0.102456	0.6967
1500	dry	Impatiens burtonii	IB	HSL	1	258.4	1.666667	1.5	0.00645	n	Weak-No	Pink	o	Present	2.9129	1.0358	0.257584	0.386376
1500	wet	Impatiens burtonii	IB	HSL	1	111.1167	15.83333	1	0	n	Weak-No	Pink	o	Present	2.9129	1.0358	0.257584	0.257584
1500	wet	Impatiens etindensis	IE	HSL	1	232.8333	5.833333	1.714286	1.15247	y	Weak-No	Red	redorange	Absent	2.027	1.848143	1.151554	1.974092
1500	wet	Impatiens mannii	IMn	HSL	2	169.525	35	1.214286	0	n	Weak-No	White	o	Present	2.27125	1.0241	0.052178	0.063359
1500	dry	Impatiens niamniamensis	IN	HSL	1	212.0833	0.833333	3	0.121807	y	Weak-No	Red	redorange	Absent	2.0146	2.1059	1.74866	5.24598
1500	wet	Impatiens niamniamensis	IN	HSL	1	238.4667	4.166667	7.6	3.040257	y	Weak-No	Red	redorange	Absent	2.0146	2.1059	1.74866	13.28982

1500	dry	Isoglossa glandulifera	IG	HSL	1	203.1167	1.666667	1	0	n	Weak-No	White	o	Present	1.808	1.229375	0.015064	0.015064
1500	dry	Ixora guineensis	IGu	T	1	24	5	61.66667	0.208333	n	Mod-strong	White	o	Absent	1.4318	1.3668	0.047823	2.949088
1500	wet	Ixora guineensis	IGu	T	1	137.9833	9.166667	151.8182	0.066433	n	Mod-strong	White	o	Absent	1.4318	1.3668	0.047823	7.260407
1500	dry	Kigelia africana	KA	T	1	64	18.33333	6.954545	54.71354	y	Weak-No	Red	redorange	Absent	7.4974	2.4106	11.00024	76.50168
1500	dry	Melanthera scandens	MS	HSL	2	197.3167	30.83333	343.9189	0	n	Weak-No	Orange	redorange	Absent	0.168	0.2417	0.002905	0.999022
1500	wet	Melanthera scandens	MS	HSL	2	197.3167	20	290.4167	0	n	Weak-No	Orange	redorange	Absent	0.168	0.2417	0.002905	0.843607
1500	dry	Mimulopsis solmsii	Mso	HSL	1	225.0833	5	1.5	0.066642	n	Mod-strong	Purple	o	Present	2.688167	1.802	0.943702	1.415552
1500	dry	Momordica foetida	MF	HSL	2	75.76667	1.666667	1	0	n	Weak-No	Yellow	o	Present	1.6882	0.4618	0.060767	0.060767
1500	wet	Momordica foetida	MF	HSL	2	75.76667	8.333333	1.3	0	n	Weak-No	Yellow	o	Present	1.6882	0.4618	0.060767	0.078997
1500	dry	Mussaenda tenuiflora	MT	HSL	1	64	0.5	24	0.421875	n	Weak-No	Yellow	o	Present	1.130667	2.916444	0.960897	23.06153
1500	wet	Mussaenda tenuiflora	MT	HSL	1	62	0.5	13	0.209677	n	Weak-No	Yellow	o	Present	1.130667	2.916444	0.960897	12.49166
1500	dry	Piper capense	PC	HSL	2	132.8833	3.333333	337.5	0	n	Weak-No	White	o	Absent	0.0838	0	0.000166	0.055957
1500	wet	Piper capense	PC	HSL	2	132.8833	4.166667	475	0	n	Weak-No	White	o	Absent	0.0838	0	0.000166	0.078755
1500	dry	Plectranthus kamerunensis	PlK	HSL	1	219.7667	3.333333	13.25	0.106173	n	Weak-No	Purple	o	Present	1.0456	0.4296	0.40062	5.308222
1500	wet	Plectranthus kamerunensis	PlK	HSL	1	88.76667	6.666667	7.75	0.45062	n	Weak-No	Purple	o	Present	1.0456	0.4296	0.40062	3.104809
1500	wet	Polystachia bicalcarata	PBi	HSL	2	22.83333	10.83333	2.923077	0	n	Weak-No	Pink	o	Present	0.6772	0.36	0.000956	0.002796
1500	dry	Psydrax dunlapii	PDu	T	1	64	6.666667	969.375	3.333333	n	Mod-strong	Yellow	o	Present	0.5325	0.358	0.086029	83.39423
1500	dry	Solanecio mannii	SoM	T	2	93.35	0.833333	1000	0	n	Weak-No	Yellow	o	Absent	0.2678	0.6144	0.005198	5.197593
1500	wet	Solanum nigrum	SN	HSL	2	36.00833	0.833333	2	0	n	Weak-No	White	o	Present	1.0004	0.3304	0.002191	0.004382
1500	wet	Spermacoce princeae	SPr	HSL	2	85.55833	90	2.046296	0	n	Weak-No	White	o	Absent	1.4016	0.4076	0.012759	0.02611
1500	dry	Stachys aculeolata	SAc	HSL	1	115	0.5	3	0	n	Weak-No	Purple	o	Present	0.4862	0.399	0.055253	0.165758
1500	wet	Stachys aculeolata	SAc	HSL	1	120.45	24.16667	2.034483	0	n	Weak-No	Purple	o	Present	0.4862	0.399	0.055253	0.112411
1500	dry	Syzygium sp 1	Ssp	T	1	64	5.833333	3435.714	61.06771	n	Mod-strong	White	o	Absent	2.0898	0.2716	0.679186	2333.49
1500	dry	Tabernemontana ventricosa	TV	T	1	64	95.83333	64.91304	277.0182	n	Mod-strong	White	o	Absent	3.645167	1.22	1.104348	71.68659
1500	dry	Thonningia sanguinea	TS	HSL	1	93.45	0.5	1	0.160514	y	Weak-No	Red	redorange	Absent	4.062182	0	50.09974	50.09974
1500	wet	Thonningia sanguinea	TS	HSL	1	230.5667	17.5	1	28.69018	y	Weak-No	Red	redorange	Absent	4.062182	0	50.09974	50.09974
1500	wet	Utricularia mannii	UM	HSL	1	115.7	10	1.083333	0	n	Weak-No	Yellow	o	Present	1.9271	0.6938	0.001231	0.001333
2200	dry	Acanthopale decempedalis	AD	HSL	1	216.8	22.5	1.296296	0.622694	n	Weak-No	White	o	Present	1.854714	2.200929	0.476316	0.617447
2200	wet	Acanthopale decempedalis	AD	HSL	1	211.3	6.666667	1.125	2.366304	n	Weak-No	White	o	Present	1.854714	2.200929	0.476316	0.535855

2200	wet	Begonia oxyanthera	BOa	HSL	2	34.60833	2.5	10.66667	0	n	Weak-No	White	o	Present	1.3856	0	0.000942	0.010051
2200	dry	Begonia poculifera	BP	HSL	2	171.3167	0.833333	2	0	n	Weak-No	White	o	Absent	2.6235	0	0.001256	0.002511
2200	wet	Begonia poculifera	BP	HSL	2	171.3167	21.66667	5.153846	0	n	Weak-No	White	o	Absent	2.6235	0	0.001256	0.006471
2200	dry	Clausena anisata	CAn	T	1	64	0.5	1181	0.445313	n	Weak-No	White	o	Absent	1.0498	0	0.082437	97.3583
2200	wet	Cyphostemma mannii	CyMa	HSL	2	63.025	1.666667	10.5	0	n	Weak-No	Green	o	Absent	0.4202	0	0.002371	0.024894
2200	wet	Disperis kamerunensis	DK	HSL	1	182.3333	2.5	2	0	n	Weak-No	White	o	Present	1.115833	0.566	0.002782	0.005564
2200	dry	Hypoestes triflora	HT	HSL	1	223.5333	30	1.388889	0.134208	n	Weak-No	White	o	Absent	1.2425	0.6914	0.185328	0.2574
2200	wet	Chassalia laikomensis	ChL	HSL	1	207.85	6.666667	7.125	0.064149	n	Mod-strong	White	o	Absent	0.832286	0.743143	0.102456	0.729998
2200	dry	Ilex mitis	IM	T	2	49.075	16.66667	17170	0	n	Mod-strong	White	o	Absent	0.6492	0.1892	0.060925	1046.084
2200	dry	Impatiens sakeriana	IS	HSL	1	218.6	1.666667	1	0.396462	y	Weak-No	Red	redorange	Absent	1.405571	1.817	3.903344	3.903344
2200	wet	Impatiens sakeriana	IS	HSL	1	160.9333	1.666667	1.5	0.807788	y	Weak-No	Red	redorange	Absent	1.405571	1.817	3.903344	5.855015
2200	dry	Isoglossa glandulifera	IG	HSL	1	198.3833	6.666667	1	0	n	Weak-No	White	o	Present	1.808	1.229375	0.015064	0.015064
2200	wet	Isoglossa glandulifera	IG	HSL	1	109.7833	0.833333	1	0	n	Weak-No	White	o	Present	1.808	1.229375	0.015064	0.015064
2200	dry	Ixora foliosa	IFo	T	1	68	10	220.8333	2.205882	n	Mod-strong	White	o	Absent	1.2332	1.2582	0.120681	26.65031
2200	wet	Jasminum preussii	JP	HSL	1	202.4	5	29.66667	0.568182	n	Mod-strong	White	o	Absent	3.885	1.913	0.185589	5.505792
2200	wet	Liparis deistelii	LD	HSL	1	167.1	0.5	2	0	n	Weak-No	Yellow	o	Absent	0.872625	0.667	0	0
2200	dry	Maesa lanceolata	ML	T	2	62.30833	5	8891.667	0	n	Weak-No	White	o	Absent	0.454429	0.032	0.004531	40.28908
2200	wet	Maesa lanceolata	ML	T	2	62.30833	0.833333	50	0	n	Weak-No	White	o	Absent	0.454429	0.032	0.004531	0.226555
2200	dry	Nuxia congesta	NC	T	1	64.05	2.5	38033.33	65.33958	n	Mod-strong	White	o	Absent	0.654857	0.459714	0.209116	7953.366
2200	wet	Piper capense	PC	HSL	2	132.8833	234.1667	723.0107	0	n	Weak-No	White	o	Absent	0.0838	0	0.000166	0.119875
2200	dry	Psydrax dunlapii	PDu	T	1	64	9.166667	334.5455	7.304688	n	Mod-strong	Yellow	o	Present	0.5325	0.358	0.086029	28.78057
2200	wet	Psychotria hypsophila	PH	HSL	1	251.4667	74.16667	6.640449	53.38348	n	Weak-No	White	o	Absent	0.528833	0.824	0.369128	2.451179
2200	dry	Schefflera abyssinica	SA	T	1	65.11667	0.833333	623922	31.28999	n	Weak-No	Green	o	Absent	0.728	0	0.260075	162266.6
2200	dry	Syzygium staudtii	SS	T	1	64	6.666667	1312.5	16.14583	n	Mod-strong	White	o	Absent	1.157	0.274333	0.85406	1120.954

* recognised life forms: T = tree, HSL = herb/shrub/liana

** datasets: 1 = observation of bird-plant interaction, 2 = data on bird visitor frequency derived from dataset 2 (observations of insect-plant interaction)

Supplementary Table 2. Summary of observed plant and bird species per season and elevation

elevation	season	# plant species visited by birds	# observed plant species	# visits	# sunbird species	# visits - sunbirds	# bird species in total
650	dry	9	29	717	9	700	10
650	wet	6	24	119	5	119	5
1100	dry	11	24	1057	5	996	7
1100	wet	9	24	202	4	202	4
1500	dry	18	28	1442	3	1433	5
1500	wet	12	29	1109	2	1109	2
2200	dry	9	13	4456	2	3600	11
2200	wet	5	13	371	2	371	2

CHAPTER IV

Bartoš M, Janeček Š, Janečková P, Padyšáková E, Tropek R, Götzenberger L, **Klomberg Y**, Jersáková J (2020) Self-compatibility and autonomous selfing of plants in meadow communities. *Plant Biology* 22(1): 120-128. (IF_{2019}: 2.156).

YK participated in collection of plants for the greenhouse experiment and was deeply involved in collecting floral trait data in the field. Additionally, YK was involved in the writing of the manuscript.

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RESEARCH PAPER

Self-compatibility and autonomous selfing of plants in meadow communities

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Keywords
Autonomous selfing; floral traits; hand-pollination; inbreeding depression; meadows; self-compatibility.

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ABSTRACT

- One of the most fundamental, although controversial, questions related to the evolution of plant mating systems is the distribution of outcrossing rates. Self-compatibility, and especially autonomous self-pollination, can become particularly beneficial in anthropogenically degraded habitats with impoverished pollinator assemblages and increased pollen limitation.
- In a hand-pollination experiment with 46 meadow plants from the Železné hory Mts., Czech Republic, we evaluated the species' ability to adopt different mating systems. For a subset of the species, we also tested seed germination for inbreeding depression. Subsequently, we analysed relationships between the species' mating systems and 12 floral and life-history traits.
- We found a relatively discrete distribution of the studied species into four groups. Fully and partially self-incompatible species formed the largest group, followed by self-compatible non-selfers and mixed mating species. The germination experiment showed an absence of inbreeding depression in 19 out of 22 examined species. Nectar sugar per flower, nectar sugar per shoot and dichogamy were significant associated with the mating system.
- Spontaneous selfing ability and self-incompatibility in species of the meadow communities had a discrete distribution, conforming to the general distribution of mating and breeding systems in angiosperms. The low frequency of spontaneous selfers and the lack of inbreeding depression at germination suggest the existence of a selection against selfing at the later ontogenetic stages. Some floral traits, such as the level of dichogamy and amount of nectar reward, may strongly impact the balance between selfing and outcrossing rates in the self-compatible species and thus shape the evolution of mating systems.

INTRODUCTION

Since the 19th century there has been an upsurge in attempts to understand the occurrence and evolution of selfing and outcrossing in plant mating systems (Darwin 1876; Stebbins 1957). Mating systems have significant consequences for the ecology, distribution and genetic structure of plant populations, and the study of reproductive modes has received considerable attention in the literature on plant evolution for decades (Fryxell 1957; Barrett 2003; Karron *et al.* 2012). Efforts to understand these phenomena have become even more urgent in recent decades as plant populations have become more and more exposed to the degradation of natural habitats, landscape fragmentation and a decline in pollinators (Kearns *et al.* 1998). Despite the long history of research, we are still far from understanding the major patterns and processes related to plant mating systems.

One of the most fundamental, although still controversial, patterns in the context of mating system evolution is the outcrossing rate distribution in nature (Holsinger 1991; Goodwillie *et al.* 2005; Charlesworth 2006). In their pioneering work, Lande & Schemske (1985) demonstrated a bimodal distribution of outcrossing rates for 55 plant species and concluded that natural selection leads to evolution of outcrossing and selfing as two discrete strategies.

Such a dichotomy was explained by decreasing fitness in primarily outcrossing populations due to inbreeding depression, whereas in selfing populations the deleterious recessive alleles were quickly excluded from a population through natural selection. Subsequent plant breeding surveys (*e.g.* Barrett & Eckert 1990; Vogler & Kalisz 2001; Goodwillie *et al.* 2005; Goodwillie *et al.* 2010) re-analysed the bimodality of plant outcrossing rates and confirmed the tendency towards bimodality in wind-pollinated plants, while outcrossing rates in animal-pollinated systems had a rather continuous character, with a considerable amount of species having mixed mating systems. This intermediate strategy between selfing and outcrossing is considered to increase plant fitness in some

temporal unfavourable conditions (*e.g.* shortage of pollinators in some years or seasons). Under such conditions, at least some basic self-pollination represents a form of reproductive assurance (Barrett 2002; Kalisz *et al.* 2004).

Nevertheless, there have been remarkably few efforts to systematically collect data on selfing ability (both autonomous and geitonogamous) and self-incompatibility (SI) across species to estimate their distribution (but see Lloyd & Schoen 1992; Raduski *et al.* 2012). Vogler & Kalisz (2001) argued that the conclusions on outcrossing distribution are sensitive to the statistical and data presentation approaches, especially whether the outcrossing rates are considered as a categorical or continuous variable. Moreover, only a small number of reviewed studies report any experimental evidence of selfing ability and self-incompatibility, the fundamental mechanisms affecting outcrossing rates in nature (Goodwillie *et al.* 2005). The relationship between mating systems is not necessarily trivial, as concluded by Lloyd & Schoen (1992). Based on analyses of published breeding systems of 66 plant species, these authors revealed a significant relationship between self-compatibility (SC) and autonomous selfing. Partially SI species had, on average, lower capacity for selfing than SC species, although some exceptions are known in both systems. Finally, the authors found a continuum between partially SI and SC plants.

Importantly, as mentioned above, the bimodality of mating systems premises that primarily self-pollinated plants are less negatively affected by an inbreeding depression. A recent literature review on inbreeding depression (ID) effects on germination of inbred and outbred seeds of 233 plant species from 64 families showed that inbred and outbred seeds germinated comparably in half of all studied cases (Baskin & Baskin 2015). Such results supported the hypothesis that partially recessive deleterious alleles responsible for ID are better purged in selfing populations (Byers & Waller 1999; Glémin & Ronfort 2013). In another review, Winn *et al.* (2011) showed lower values of ID for selfers compared to outcrossers, consistent with the theoretical expectation of purging of genetic load and in accordance with previous surveys. Husband & Schemske (1996) found that selfers and outcrossers may also differ in the timing of ID, *i.e.* outcrossers are expected to be more susceptible to ID (and especially to early ID) than selfers.

Moreover, selfing ability and SI need to be considered as influenced by floral traits, which are often under correlational selection, *i.e.* that one floral trait influences the selection pressure of another (Sletvold & Ågren 2011; Campbell *et al.* 2014; Chapurlat *et al.* 2015). Consequently, individual floral traits can be in a mutual correlation, and the specific trait combinations can thus be phylogenetically conserved. No or low selfing ability is more frequent in plants with specialised pollination mechanisms, often characterised by bilaterally symmetrical flowers, fused petals and relatively few stamens per flower (Lloyd & Schoen 1992). These plants usually possess mechanisms promoting outcrossing, such as a strong spatial (herkogamy; Karron *et al.* 1997; Motten & Stone 2000; Haddadchi & Fatemi 2015) or temporal (dichogamy; Brys *et al.* 2013) separation of sexual organs. Conversely, the species with high autonomous selfing often possess radially symmetrical flowers with reduced floral display, separate petals and more than five stamens per flower (Lloyd & Schoen 1992; Goodwillie *et al.* 2010; Brys *et al.* 2013). Nevertheless, both genetic and phylogenetic data suggest frequent transitions from SI to SC (Goldberg *et al.* 2010; Wright *et al.* 2013).

Furthermore, the evolution of SI can also be affected by the foraging behaviour of pollinators, which influences outcross- and self-pollen deposition, and thereby the ecological selfing rates of plants (Devaux *et al.* 2014). Floral traits involved in pollinator attraction, such as high nectar production (Johnson *et al.* 2004; Fisogni *et al.* 2011) and mass flowering of clonally propagating species (Eckert 2000; Hu *et al.* 2015), strongly increase the probability of self-pollination among flowers of the same plant (*i.e.* geitonogamous selfing). Although clonal plants can be predicted as rather SI, as suggested by a study on Australian *Droseraceae* (Chen *et al.* 1997), SI or SC association with clonality seems to be dependent also on a particular clonal strategy, as documented by comparative studies of the closely related species *Eichhornia azurea* and *E. crassipes* (Barrett 2015 and citations therein). Geitonogamy is often considered a consequence of traits related to higher plant attractiveness, *e.g.* high nectar production or number of flowers (de Jong *et al.* 1993). In contrast, traits correlated with autonomous selfing are much more refined, and plants with a high ability for autonomous selfing are expected to invest less in pollinator attraction and reward (Eckert *et al.* 2010; Thomann *et al.* 2013). These expectations may be especially valid for prior selfing (*i.e.* not mediated by pollinators and occurring before outcrossing), whereas competing or delayed selfing, which occur during and after pollinator visitation, likely maintain the floral traits promoting outcrossing (Kalisz & Vogler 2003; Lepers *et al.* 2014).

In this study, we present and analyse a comprehensive experimental dataset on the extent of SC (*i.e.* ability to produce seeds after self-pollination) and ability for spontaneous selfing in 46 plant species growing in meadows in a fragmented cultural landscape in Central Europe. For a subset of these species, we also tested seed germination to reveal inbreeding depression. Finally, we analysed the relationship of SC and selfing ability with a wide spectrum of floral traits. We aimed to answer the following questions: (i) do individual mechanisms contributing to the outcrossing rate in nature (*i.e.* ability for selfing and SC) represent a discrete distribution or a continuum; (ii) are plant species with predominantly outcrossing mating systems more negatively affected by inbreeding depression; and (iii) which floral traits are correlated with selfing ability and SC?

MATERIAL AND METHODS

Pollination experiment

The pollination experiment involved 46 plant species collected from semi-natural wet meadows in the Železné hory Protected Landscape area (part of the Bohemian-Moravian Highlands, Czech Republic; 49°48′2.74″ N, 15°43′56.57″ E) during the 2016 and 2017 vegetation seasons. These oligotrophic to mesotrophic meadows represent remnants of formerly common wetlands, nowadays often drained and/or changed into arable and cultivated fields. At least 12 individuals of each species from no less than three different populations were transplanted into individual pots and placed in outdoor containers at the Institute of Botany of the Czech Academy of Sciences in Třeboň at the beginning of each growing season. The plants were grown in soil mixtures composed of commercial

horticultural substrate, sand and peat with a ratio of 2:2:1. The pollination experiment itself was carried out in a greenhouse, into which the plants were gradually moved just before flowering. To prevent any natural pollination, the plants were enclosed in large mesh cages (105 × 160 × 160 cm). For hand-pollination, we chose plants with at least three freshly opened flowers and applied three treatments to each plant: (1) cross-pollination with pollen from another population, (2) geitonogamous self-pollination with pollen from the same plant to test for self-compatibility, and (3) no manipulation to test for autonomous selfing. Inside the cages, the pots (15 × 15 cm) with individual plants were spatially separated (minimum distance between pots was 20 cm) to prevent contact between flowers and minimise possible contamination from inappropriate pollen during manipulations. The flowers were hand-pollinated with fresh pollen using a brush or cotton swabs each day during the entire flowering period to ensure successful pollination. Later, developing ovaries were covered with small fine mesh nylon bags to prevent seed loss. Well-developed seeds were counted, and the number of seeds served as an indicator of the efficiency of a particular treatment. To avoid potential technical problems during the pollination and other manipulations from flowers in compact inflorescences (such as a capitulum in *Asteraceae*), the treatments were applied to the whole flowerhead of three inflorescences per plant. In *Selinum carvifolia*, with compound umbels, we assigned the treatments to three umbellets. In *Anemone nemorosa*, the treatments were applied to three individual plants, as this species produces just a single flower per shoot. Nevertheless, in our study, as well as in the majority of studies, the ability for apomixis (*i.e.* asexual seed production) is overlooked, although it is relatively common in perennial plants (Richards 2003). However, this feature was previously documented only for two species (*Ranunculus acris* and *R. auricomus*) in our dataset (Harper 1957; Hörandl 2008).

Germination experiment

Seeds harvested from the pollination experiment in 2016 were sown on sterilised sand (80 °C for 1 h) in 60- or 90-mm large Petri dishes. Each dish was divided into three segments to accommodate seeds from three treatments applied to individual plants. The number of dish replicates per plant species (corresponding to the number of treated individuals) ranged from six to 12. All available seeds, but maximum 25 seeds per treatment, and seeds of individual plants were sown. The dishes were cultured in an air-conditioned room at 22 °C and an 8-h light/16-h dark period. The experiment ran from the beginning of February to the end of April 2017. Irrigation was performed by slowly and evenly dripping water onto the sand with a pipette. The pots were regularly observed, the numbers of emerging seedlings in each pot were counted and these were removed from the dishes. For statistical analyses, we used 22 plant species for which we had at least one germinating seed in no less than half of the replicates.

Floral and life-history traits

To explore the effect of floral and life-history traits on the mating system of target plant species, we compiled floral and life-history data from available databases (Biolflor, Klotz *et al.* 2002; Clo-Pla, Klimešová *et al.* 2017) and our field measurements of 12 traits. An overview of traits and methods used for their measurements are shown in Table 1.

Statistical analyses

First, we sorted the plant species into four groups according to their mating system, based on comparison of the number of outcrossed seeds (O) with geitonogamous (G) and autogamous (A) seed production (Fig. 1A). We assume that outcrossing results in the maximum number of seeds possible, therefore A/O and G/O ratios were used as proxies for the extent of selfing and self-compatibility in interspecific comparisons. Clustering of species in a scatter plot was tested using the Clark–Evans test ($R = 0.780$, $P = 0.002$) of aggregation for a spatial point pattern (Clark & Evans 1954). This returns the ratio (R) of the observed mean nearest neighbour distance in the pattern to that expected for a Poisson point process of the same intensity. An R value >1 suggests regular ordering, while R value < 1 suggests clustering (Clark & Evans 1954). The first group consisted of highly self-incompatible species with limited autonomous selfing (*SI non-selfers*), the second group comprised highly self-compatible species with limited autonomous selfing (*SC non-selfers*) and the third group combined highly self-compatible species capable of substantial autonomous selfing (*SC selfers*). The last group consisted of five species with mixed mating. The data from pollination and germination experiments contained many zeros, *i.e.* no seeds developed or germinated, and therefore they did not meet normality assumptions even after transformation. Hence, we used the non-parametric permutation ANOVA in the PERMANOVA + for PRIMER program for the intraspecific comparison of seed production after the pollination treatments (Anderson *et al.* 2008).

Consequently, we conducted one-way ANOVA (numerical traits) or Chi-square tests for contingency tables (categorical traits) to examine associations between the floral traits and the species' mating system. The strength of the association between categorical variables was expressed as Cramer's V, with a range from 0 for no association to 1 for a strong association (Cramer 1946). Because some cells had low numbers of expected frequencies, we also conducted Fisher's exact test; the results were consistent with the previous tests. For the numerical traits, we performed *post-hoc* tests to compare means of the four mating system groups.

In addition, we assessed whether any phylogenetic signal was present in the residuals of the ANOVA models, allowing us to infer the evolutionary independence of the studied associations. For this purpose, we applied Pagel's lambda (Pagel 1999) and Blomberg's K (Blomberg *et al.* 2003). Because these tests of model residuals cannot be applied on categorical variables, for categorical traits we assessed the residuals of ANOVA models where we set up models with G/O and A/O as a response (instead of testing the mating system as one of the four categories) and the categorical variables as an explanatory variable. Phylogenetic information for all considered species was extracted from the DaPhnE phylogenetic tree (Durka & Michalski 2012).

RESULTS

The most frequent mating system of the studied plants of meadow communities was represented by *SI non-selfers* (20

Table 1. Description of measured floral and life-history traits.

Trait	definition (method, units)	type of variable	source
Start of flowering	Start of flowering phase (month)	Quantitative	Biolflor
Dichogamy	Temporal separation of generative organs (transformed from the original 7 categories to a continuous variable ranging from 0 to 0.5, with the upper value denoting an absence of dichogamy, *i.e.* simultaneous presence of male and female organs)	Quantitative	Biolflor
Nectar sugar per flower	Daily nectar production (a flower in 15 individuals per plant species bagged at full anthesis for 24 h in the field; nectar washed into distilled water with a 100-μl Hamilton syringe and stored in a refrigerator prior to freezing following Morrant *et al.* (2008); amount of nectar sugars quantified by HPLC using the ICS-3000 system (Dionex), with an electrochemical detector and CarboPac PA 1 column; nectar production expressed in milligrams of nectar sugars per flower per day)	Quantitative	This study
Nectar sugar per shoot	Nectar sugar per flower multiplied by number of open flowers per shoot	Quantitative	This study
No. of open flowers per shoot	Number of simultaneously open flowers on a shoot (counted on 60 ind. per species at 3 meadows)	Quantitative	This study
No. of ovules per flower	Counted on dissected ovaries of min. 6 ind. from 3 meadows	Quantitative	This study
No. of stamens per flower	Counted on flowers of min. 6 ind. from 3 meadows	Quantitative	This study
No. of ovules per shoot	No. of ovules per flower multiplied by number of open flowers per shoot	Quantitative	This study
No. of stamens per shoot	No. of stamens per flower multiplied by number of open flowers per shoot	Quantitative	This study
Clonal multiplication	Number of vegetative offspring per maternal shoot per year	Quantitative	Clo-Pla
Flower symmetry	Actinomorphic or zygomorphic	Categorical	This study
Flower types	Structural blossom classes following Faegri & van der Pijl (1979) – umbel, bowl, head, brush, bell, tube, gullet and flag	Categorical	This study

species), followed by *SC non-selfers* (13 species) and *SC selfers* (eight species) Only five species represented a *mixed mating* system (Fig. 1A). The seed production from autonomous selfing was always lower or equal to the geitonogamous and outcrossed treatments, except for *Trifolium repens*, with twice as many seeds produced by autonomous selfing than by the other two treatments (Table 2).

Inbreeding depression expressed at the germination stage was not statistically significant in most of the 22 species tested (Table 3). Substantially lower germination after the geitonogamous selfing treatment was found in only two *SC non-selfers* (*Cardamine amara* and *Lychnis flos-cuculi*). One largely SI species, *Crepis paludosa*, had a higher germination of the geitonogamous seeds than the outcrossed seeds.

Among the tested quantitative floral trait variables, nectar sugar per flower, nectar sugar per shoot and dichogamy showed significant associations with the mating system (Table 4). The *SI non-selfers* presented significantly less nectar sugar per flower and per shoot than *SC non-selfers* (Fig. 1B, 1). The former group also possessed less pronounced temporal segregation of male and female reproductive structures than the latter group (Fig. 1D). The third group, *SC selfers*, showed values similar to the other groups; only in the case of nectar sugar per shoot were the values significantly lower than in *SC non-selfers* (Fig. 1C). We present all mutual correlation indices of particular quantitative floral traits in Table S1.

While residuals of the models relating mating system to dichogamy and nectar sugar per flower did not show any significant phylogenetic signal in terms of lambda (dichogamy: lambda = 0.336, $P = 0.342$; nectar sugar per flower: lambda 0, $P = 1.000$) or Bloomberg's K (dichogamy: $K = 0.095$, $P = 0.268$; nectar sugar per flower: $K = 0.088$, $P = 0.256$), residuals of the model with nectar sugar per shoot showed phylogenetic signal for both metrics (lambda = 0.835, $P = 0.006$; $K = 0.214$, $P = 0.012$). No significant phylogenetic signal was found for the residuals in the ANOVAs for G/O and A/O as separate response variables.

DISCUSSION

Selfing ability and compatibility

Our experimental hand-pollination of 46 plant species from meadows of Central Europe represents a valuable dataset on selfing ability and SI at the community level. The examined plant species clustered into three discrete groups by autonomous selfing and SC levels, supplemented by a few species with mixed mating that were not clearly affiliated to any of the three main groups (Fig. 1A). Similar to Lloyd & Schoen (1992), a large proportion of the highly SI plants had a very low capacity for autonomous selfing. Highly SC species clustered into two well-separated groups, depending on their ability to self spontaneously. While Lloyd & Schoen (1992) found no obvious boundary separating partially SI and SC plants, our data better fit to a bimodal distribution of SI, as also reported by Raduski *et al.* (2012). In their compiled study of 1,200 angiosperm species, 31% were SC ($SI \leq 0.2$), 25% were partially SI ($0.2 < SI < 0.8$) and 44% were SI ($SI \geq 0.8$). Applying their simplified distribution of breeding systems into the three discrete states, our dataset would contain 26% SC, 44% partially SI and 30% SI species. However, the last category would increase to 41% if the threshold for SI were lowered to 0.7, *i.e.* the threshold commonly used to distinguish SI species (*e.g.* Matallana *et al.* 2010).

If we consider the distribution of mating systems at a community level, the dominance of highly SI plants in our meadow communities corresponds well to the relatively high proportion of outcrossers predicted for stable environments with many highly competitive plant species (Munoz *et al.* 2016).

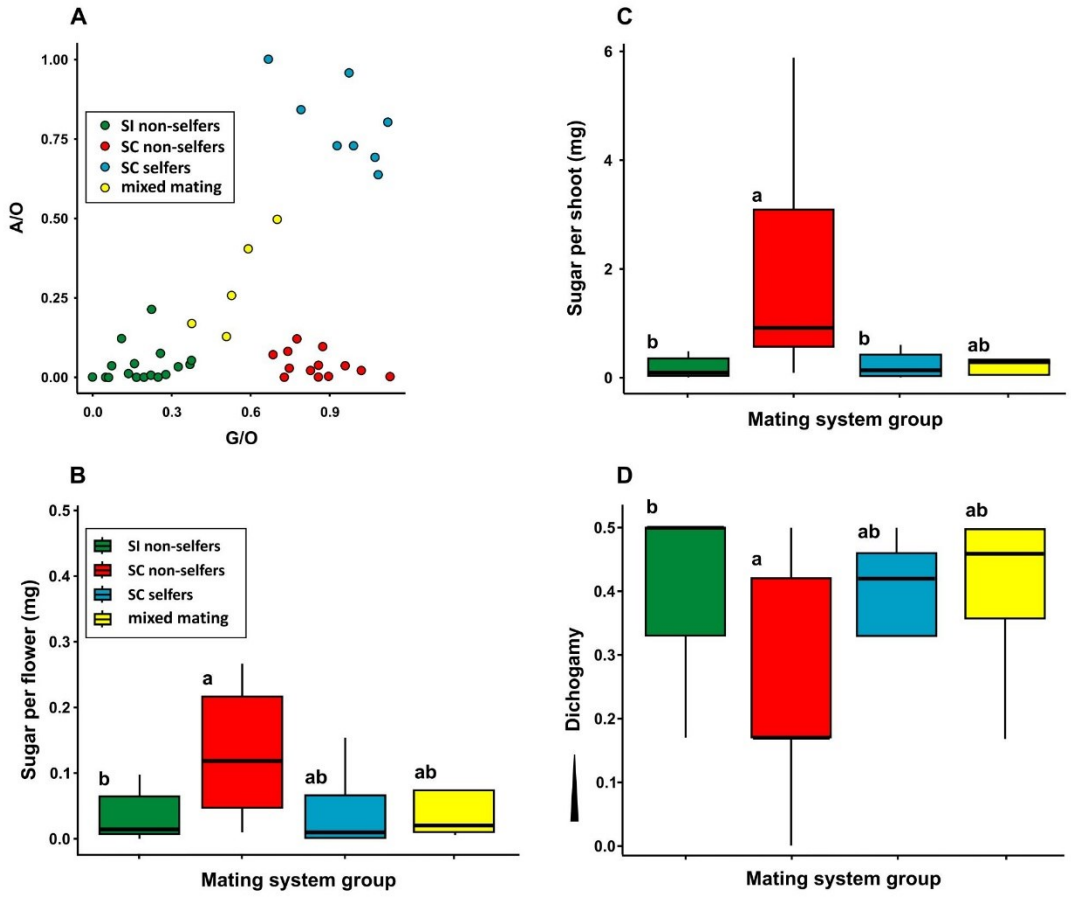

Fig. 1. (A) Scatterplot of A/O and G/O in combination with the results of the cluster analysis depicting four mating system groups. (B–D) Amount of nectar sugar per flower, per shoot and extent of dichogamy in the four mating system groups; individual letters denote significant results of *post-hoc* comparisons. Medians (solid line), first and third quartiles (boxes), marginal values >1.5 times the inter-quartile range (whiskers) are visualised. In (D), 0.5 on the *y*-axis denotes an absence of dichogamy, *i.e.* simultaneous presence of male and female organs. See text for details of models and their results

Inbreeding depression at germination

In our germination experiment, only three out of the 22 examined species showed significant differences between the outcrossed and selfed seeds (Table 3), supporting the lack of differences between germination of inbred and outbred seeds in half of the 743 case studies reviewed by Baskin & Baskin (2015). Unfortunately, we did not estimate ID in later ontogenetic stages to test differences between selfers and outcrossers in the timing of ID (Husband & Schemske 1996). However, because outcrossers are expected to be more susceptible to ID (especially to early ID), potential differences among the species should manifest already early in the life cycle (Richards 2000; Angeloni *et al.* 2011). We also have not found any support for the higher inbreeding depression in SI species compared to SC species (Voillemot & Pannell 2017). However, there could be a huge range of variation in the magnitude of ID for seed germination that may depend on many factors, *e.g.* physical environment or degree of competition under which seeds were produced, outcrossing distance, ploidy level and dormancy-breaking treatment (Baskin & Baskin 2015).

Correlation with floral traits

Among the analysed floral traits, only nectar sugar content per flower and shoot, and dichogamy significantly correlated with the grouping of plants according to their selfing ability and SC. In general, increased nectar volume or sucrose reward positively influence visitation rates of pollinators, as well as the duration of visits (*e.g.* Pappers *et al.* 1999; Roldán-Serrano & Guerra-Sanz 2005). However, the higher attractiveness to pollinators often translates into higher pollen discounting due to pollinator-mediated geitonogamous selfing (Jersakova & Johnson 2006). In our study, the SC plants with limited ability for spontaneous selfing produced the most nectar sugar (Fig. 1B, 1). Since the SC plants may suffer less from the consequences of selfing (Voillemot & Pannell 2017), pollinator-mediated geitonogamy in the SC species could increase reproductive assurance

Table 2. Mean number of seeds per flower (or inflorescence*).

Species	F_{perm}	*P*-value	$A_{(mean)}$	$G_{(mean)}$	$O_{(mean)}$	G/O	A/O
SI non-selfers							
Anemone nemorosa	24.45	**0.000**	0.57^{a}	5.57^{b}	17.14^{c}	0.32	0.03
Caltha palustris	11.07	**0.001**	19.3^{a}	20.2^{a}	90.25^{b}	0.22	0.21
Campanula patula	10.93	**0.000**	1.5^{a}	46.33^{a}	209.17^{b}	0.22	0.01
Cardamine pratensis	33.46	**0.000**	0.0^{a}	0.71^{a}	14.5^{b}	0.05	0.00
*Crepis paludosa**	186.77	**0.000**	1.33^{a}	4.92^{b}	30.83^{c}	0.16	0.04
Galium boreale	169.00	**0.000**	0.0^{a}	0.0^{a}	1.86^{b}	0.00	0.00
Galium palustre	88.05	**0.000**	0.0^{a}	0.36^{a}	1.85^{b}	0.19	0.00
Galium uliginosum	149.58	**0.000**	0.0^{a}	0.0^{a}	1.86^{b}	0.00	0.00
Hypericum maculatum	29.65	**0.000**	2.22^{a}	65.73^{b}	237.0^{c}	0.28	0.01
Lathyrus pratensis	4.03	**0.037**	0.0^{a}	0.57^{ab}	2.29^{b}	0.25	0.00
Lysimachia vulgaris	25.01	**0.000**	2.92^{a}	9.92^{a}	38.5^{b}	0.26	0.08
Myosotis palustris	35.24	**0.000**	0.0^{a}	0.0^{a}	2.83^{b}	0.00	0.00
Potentilla erecta	51.23	**0.000**	0.08^{a}	0.92^{a}	6.75^{b}	0.14	0.01
Primula elatior	69.26	**0.000**	0.0^{a}	6.64^{a}	40.0^{b}	0.17	0.00
Ranunculus acris	32.71	**0.000**	0.64^{a}	1.27^{a}	17.55^{b}	0.07	0.04
Ranunculus flammula	42.58	**0.000**	1.83^{a}	13.0^{b}	34.67^{c}	0.37	0.05
Symphytum officinale	122.50	**0.000**	0.0^{a}	0.0^{a}	2.33^{b}	0.00	0.00
*Tephroseris crispa**	35.49	**0.000**	2.88^{a}	26.89^{b}	72.63^{c}	0.37	0.04
Trifolium pratense	10.29	**0.001**	0.1^{a}	0.09^{a}	0.82^{b}	0.11	0.12
Veronica chamaedrys	62.97	**0.000**	0.0^{a}	0.64^{a}	10.46^{b}	0.06	0.00
SC non-selfers							
Ajuga reptans	43.00	**0.000**	0.0^{a}	3.0^{b}	3.5^{b}	0.86	0.00
Cardamine amara	15.93	**0.000**	0.92^{a}	8.42^{a}	11.36^{b}	0.74	0.08
*Cirsium oleraceum**	28.14	**0.000**	2.1^{a}	96.73^{b}	94.82^{b}	1.02	0.02
*Cirsium palustre **	11.52	**0.001**	4.64^{a}	29.67^{b}	38.25^{b}	0.78	0.12
Geum rivale	9.73	**0.000**	2.6^{a}	23.47^{b}	26.87^{b}	0.87	0.10
Glechoma hederacea	81.11	**0.000**	0.0^{a}	3.58^{b}	3.17^{b}	1.13	0.00
Lycopus europaeus	18.56	**0.000**	0.1^{a}	2.57^{b}	3.44^{b}	0.75	0.03
Lychnis flos-cuculi	23.95	**0.000**	2.08^{a}	82.08^{b}	99.46^{b}	0.83	0.02
Scrophularia nodosa	8.34	**0.004**	0.25^{a}	98.13^{b}	109.5^{b}	0.90	0.00
*Selinum carvifolia**	15.97	**0.000**	2.25^{a}	21.83^{b}	31.92^{b}	0.68	0.07
Stachys sylvatica	18.42	**0.000**	0.08^{a}	2.08^{b}	2.17^{b}	0.96	0.04
Stellaria graminea	7.50	**0.003**	0.0^{a}	2.4^{b}	3.3^{b}	0.73	0.00
*Succisa pratensis**	26.09	**0.000**	2.0^{a}	44.92^{b}	52.42^{b}	0.86	0.04
SC selfers							
Cerastium holosteoides	0.11	0.897	40.20	40.91	42.00	0.97	0.96
Filipendula ulmaria	1.38	0.274	3.64	4.64	5.00	0.93	0.73
Impatiens parviflora	0.52	0.601	1.33	1.25	1.58	0.79	0.84
Ranunculus auricomus	1.76	0.196	5.67	9.67	8.92	1.08	0.64
Scutellaria galericulata	6.88	**0.004**	2.42^{a}	3.75^{b}	3.5^{b}	1.07	0.69
Trifolium repens	5.47	**0.029**	2.4^{a}	0.8^{b}	1.2^{ab}	0.67	2.00
Veronica officinalis	2.27	0.134	8.11	11.33	10.11	1.12	0.80
Veronica serpyllifolia	2.74	0.092	41.41	56.36	56.91	0.99	0.73
intermediate species							
Epilobium palustre	5.79	**0.010**	40.83^{a}	57.42^{ab}	82.08^{b}	0.70	0.50
Potentilla palustris	12.71	**0.000**	10.31^{a}	40.54^{b}	79.92^{c}	0.51	0.13
Prunella vulgaris	7.97	**0.005**	1.44^{a}	2.1^{a}	3.56^{b}	0.59	0.40
Ranunculus repens	40.99	**0.000**	4.0^{a}	8.89^{b}	23.58^{c}	0.38	0.17
*Sanguisorba officinalis**	14.44	**0.000**	11.78^{a}	24.11^{b}	45.78^{c}	0.53	0.26

Results of PERMANOVA are shown. The superscript letters mean significant differences of post-hoc comparisons among individual mating systems. Statistically significant P-values are in bold. Mean values of autogamous (A), geitonogamous (G) and outcrossed (O) seeds and their proportions (G/O, A/O) per species are shown.

in pollinator-limited environments or years (Karrenberg & Jensen 2012). Lower nectar rewards found in SI non-selfers and SC selfers likely decrease undesirable incompatible pollen depositions (Klinkhamer & de Jong 1993) and save energetic cost in conditions of regular self-pollination, respectively.

Dichogamy was most pronounced in the SC plants with limited ability for spontaneous selfing. As the higher content of sugar reward found in these plants likely promotes self-pollination mediated by pollinators (see above), stronger dichogamy might prevent self-pollen depositions and thus pollen

Table 3. Percentage of germinated seeds in geitonogamous and outcrossed treatments.

	Geitonogamous			Outcrossed				
Species	No. dishes	No. seeds per dish (mean ± SE)	Percentage germinated seeds (mean ± SE)	No. dishes	No. seeds per dish (mean ± SE)	Percentage germinated seeds (mean ± SE)	F_{perm}	*P*
SI non-selfers								
Caltha palustris	8	15.5 ± 3.6	1.0 ± 0.6	7	23.1 ± 1.8	17.7 ± 8.9	4.25	0.099
Crepis paludosa	10	5.9 ± 2.3	48.8 ± 14.9	12	24.1 ± 0.6	11.7 ± 6.4	7.84	0.022
Hypericum maculatum	10	22.3 ± 1.9	88.2 ± 5.0	11	24.2 ± 0.7	83.3 ± 8.6	0.19	0.66
Potentilla erecta	5	2.2 ± 0.9	50.0 ± 22.3	11	7.3 ± 0.5	36.6 ± 9.8	1.86	0.251
Ranunculus acris	7	1.8 ± 0.4	92.8 ± 7.1	11	16.4 ± 2.4	90.9 ± 3.8	0.91	0.412
Ranunculus flamula	12	11.2 ± 2.4	95.1 ± 2.8	12	24.2 ± 0.7	94.8 ± 2.1	0.02	0.893
Tephroseris crispa	9	15.3 ± 3.2	34.6 ± 12.7	8	22.5 ± 2.5	52.5 ± 10.4	0.61	0.444
SC non-selfers								
Ajuga reptans	10	3.6 ± 0.2	15.0 ± 6.6	12	3.5 ± 0.2	36.1 ± 8.0	3.8	0.084
Cardamine amara	11	8.7 ± 1.7	40.3 ± 11.0	10	11.3 ± 2.2	74.5 ± 9.8	10.98	0.012
Cirsium oleraceum	10	25.0 ± 0.0	34.4 ± 7.9	11	22.8 ± 2.2	27.6 ± 8.0	0.25	0.609
Cirsium palustre	8	21.6 ± 1.5	73.5 ± 9.4	12	21.1 ± 2.1	78.8 ± 9.1	0.09	0.78
Geum rivale	9	21.6 ± 2.3	34.3 ± 10.8	14	16.5 ± 2.1	46.8 ± 8.9	2.02	0.22
Glechoma hederacea	12	3.5 ± 0.1	42.3 ± 9.5	11	3.3 ± 0.2	24.2 ± 7.9	2.27	0.17
Lychnis flos-cuculi	12	24.0 ± 0.7	55.8 ± 11.7	11	24.2 ± 0.7	85.7 ± 5.4	5.54	0.038
Stellaria graminea	9	2.6 ± 0.6	85.7 ± 7.4	8	3.7 ± 1.1	98.7 ± 1.2	4.55	0.092
Succisa pratensis	12	19.5 ± 2.2	49.1 ± 7.1	12	23.0 ± 2.0	44.0 ± 7.5	0.33	0.57
SC selfers								
Cerastium holosteoides	10	23.70 ± 1.3	99.2 ± 0.5	10	24.4 ± 0.6	100.0 ± 0.0	1	0.622
Veronica serpyllifolia	11	25.0 ± 0.0	100.0 ± 0.0	11	25.0 ± 0.0	100.0 ± 0.0	ns	ns
intermediate species								
Epilobium palustre	10	20.6 ± 2.9	79.7 ± 7.4	12	25.0 ± 0.0	81.3 ± 5.7	0.09	0.771
Potentilla palustris	12	23.1 ± 1.6	30.1 ± 6.8	13	23.3 ± 1.3	33.0 ± 6.3	0.25	0.63
Prunella vulgaris	7	3.0 ± 0.4	67.8 ± 17.8	9	3.5 ± 0.1	87.0 ± 5.2	0.01	0.945
Ranunculus repens	9	8.2 ± 2.7	50.3 ± 11.2	11	22.7 ± 1.2	43.4 ± 11.3	0.41	0.536

The number of dishes per species by treatment, the number of seeds sown per dish, percentage of germinated seeds per species, results of PERMANOVA showing the regression estimate and *P*-value.

discounting. Protandry (stamens mature before stigma) should be more common among the SI species to reduce the deleterious effects of selfing, while the SC species are characterised by protogyny (stigma matures before stamens; Bertin & Newman 1993; Barrett 2003). However, in our dataset, 26 of 41 studied species had some level of dichogamy and the majority were protandrous. The common presence of both protandry and higher sugar reward in the SC non-selfers could help to maintain a balance between selfing and outcrossing rates in unpredictable environments.

Table 4. Associations between mating system and floral/life-history trait variables.

	traits	*F*-value	R^2	*P*
Quantitative variables	Start of flowering	1.17	0.01	0.333
	Dichogamy	3.93	0.17	**0.015**
	Nectar sugar per flower	3.99	0.17	**0.014**
	Nectar sugar per shoot	5.48	0.25	**0.003**
	No. of open flowers per shoot	0.83	0.01	0.484
	No. of stamens per flower	0.76	0.02	0.526
	No. of stamens per shoot	0.03	0.05	0.772
	No. of ovules per flower	0.12	0.06	0.946
	No. of ovules per shoot	0.72	0.02	0.549
	Clonal multiplication	0.83	0.01	0.484
		Chi-square	Cramer's V	*P*
Categorical variables	Flower symmetry	6.04	0.36	0.110
	Flower type	19.17	0.37	0.575

For quantitative variables the regression estimate, R^2, and *P*-value are shown. For categorical variables, Chi-square, Cramer's V and *P*-value are shown. Variables that had at least a marginally significant association ($P < 0.1$) are in bold.

The revealed correlations of the floral traits with a particular mating system seem to be independent of the phylogeny of the studied plant species. A significant phylogenetic signal was only found for nectar sugar per shoot, which may be caused by the dependence of this trait on the total number of flowers per shoot. It corresponds with studies documenting the phylogenetic association of the outcrossing rate and flower size/number of plant species (Sargent *et al.* 2007; Goodwillie *et al.* 2010).

CONCLUSIONS

Spontaneous selfing ability and SI systems of the meadow plant community had a discrete distribution, conforming to the general distribution of mating and breeding systems in angiosperms repeatedly reported in recent metanalyses of case studies. SI and partially SI species represented the most abundant plant groups, although the exact affiliation to the mating system depended on the arbitrary threshold of self-incompatibility indices. The low frequency of spontaneous selfers,

together with the lack of inbreeding depression at germination, suggest selection against selfing is likely acting at later ontogenetic stages. Our results indicated the possible importance of floral traits, such as level of dichogamy and amount of nectar reward, in shaping selfing and outcrossing rates in SC species through interplay with pollinators. From the conservation point of view, high relative proportions of SI species and inability to self spontaneously may represent unfavourable mating systems in small fragmented plant populations under the current scenario of declining pollination service in natural ecosystems. Future studies should focus on variability and shifts in distribution of mating systems and their resilience to adverse anthropogenic landscape changes.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Inter-correlations of traits.

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Supplementary information

Supplementary table S1. Inter-correlations of traits. Significant values at p-level < 0.05 are marked in red.

Traits	*Start of flowering*	*Dichogamy*	*Nectar sugar per flower*	*Nectar sugar per shoot*	*No. of open flowers per shoot*	*No. of stamens per flower*	*No. of stamens per shoot*	*No. of ovules per flower*	*No. of ovules per shoot*
Dichogamy	**-0.3034**								
Nectar sugar per flower	0.0450	-0.1900							
Nectar sugar per shoot	0.2840	**-0.4619**	**0.3565**						
No. of open flowers per shoot	**0.4176**	**-0.3631**	-0.1159	**0.5453**					
No. of stamens per flower	-0.2495	0.2344	0.0545	-0.2047	-0.1575				
No. of stamens per shoot	**0.3472**	-0.1785	-0.1259	**0.3347**	**0.7962**	0.0502			
No. of ovules per flower	0.1548	-0.0153	0.2466	-0.0134	-0.1584	**0.4947**	-0.0855		
No. of ovules per shoot	**0.3088**	0.0090	-0.0120	0.0950	0.1946	0.2286	0.2173	**0.6630**	
Clonal multiplication	0.2796	0.2212	-0.1104	-0.2232	-0.1695	0.0174	-0.1125	0.1384	0.2177

CHAPTER V

Bartoš M, Janeček Š, Janečková P, Padyšáková E, Tropek R, Götzenberger L, **Klomberg Y**, Jersáková J (2020) Are reproductive traits related to pollen limitation in plants? A case study from a Central European meadow. *Plants* 9(5): 640. (IF_{2019}: 2.632).

YK participated in the fieldwork, was deeply involved in collecting floral trait data and contributed to writing of the manuscript.

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Are Reproductive Traits Related to Pollen Limitation in Plants? A Case Study from a Central European Meadow

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Abstract: The deficiency of pollen grains for ovule fertilization can be the main factor limiting plant reproduction and fitness. Because of the ongoing global changes, such as biodiversity loss and landscape fragmentation, a better knowledge of the prevalence and predictability of pollen limitation is challenging within current ecological research. In our study we used pollen supplementation to evaluate pollen limitation (at the level of seed number and weight) in 22 plant species growing in a wet semi-natural meadow. We investigated the correlation between the pollen limitation index (PL) and floral traits associated with plant reproduction or pollinator foraging behavior. We recorded significant pollen limitation for approximately 41% of species (9 out of 22 surveyed). Seven species had a significant positive response in seed production and two species increased in seed weight after pollen supplementation. Considering traits, PL significantly decreased with the number of pollinator functional groups. The relationship of PL with other examined traits was not supported by our results. The causes of pollen limitation may vary among species with regard to (1) different reproductive strategies and life history, and/or (2) temporary changes in influence of biotic and abiotic factors at a site.

Keywords: pollen limitation; supplemental hand-pollination; seed number; seed weight; floral traits; wet meadow

1. Introduction

Pollen limitation (i.e., limitation of seed production by deposition of pollen grains) is among the key factors affecting the fitness of individual plants and consequently, population dynamics and species survival [1]. Therefore, with the global pollination crisis [2,3], pollen limitation has become a key topic of ecology and conservation of plant communities [4–6]. Despite several decades of research, there is still no consensus on how widespread pollen limitation is in plant communities. The optimality theory [7] and sexual selection theory [8], but see [9] predict that pollen limitation should be rare. However, numerous empirical studies showed pollen limitation as a relatively common phenomenon [10,11]. A review of 306 plant species found evidence of pollen limitation (within an individual site) in 73% of the studies [12]. Consequently, this suggested insufficient pollen receipt to

be the major cause of reduced fruit production [12]. Nevertheless, the existing geographical bias of available detailed data [13] limit any strong generalizations on the extent of pollen limitation, as well as causes and consequences in individual plant species and in communities.

Pollen supplementation experiments represent a standard method for pollen limitation quantification [14]. Based on saturation by manually applied additional pollen to flowers, it allows a robust subsequent comparison of their fruit sets and/or seed sets with naturally pollinated flowers [14]. Besides the effects of pollen saturation on the quantitative characteristics, possible trade-offs in resource allocation can be evaluated also by a qualitative comparison of seed or fruit sets (e.g., by their size or weight, [15]). Nevertheless, published results from pollen supplementation experiments are predominantly based on single-species case studies. Therefore, they may not be representative of the realized pollen limitation in communities [16]. Plant species, as well as individuals in the population, may not be equally sensitive to changes in environmental and associated biotic conditions because the possible lack of pollination depends on the ecological context, plant life history, and type of breeding system [1].

The shift of plant species to outcrossing can be caused by specific plant trait evolution regardless of the possible consequence of pollen limitation [12]. However, the correlation of pollen limitation with various life-history and ecological traits was tested in only a few comparative studies [11,17]. In 224 species from 64 families of flowering plants, Larson and Barrett [11] revealed pollen limitation as less intense in species which are self-compatible, autogamous, monocarpic, herbaceous, nectariferous, and occurring in open habitats and temperate regions. Although self-incompatible plants are generally expected to be more pollen limited than self-compatible plants [10], this assumption may not always be true. As discussed by García-Camacho and Totland [17], self-compatible species may potentially receive more compatible pollen on their stigmas than self-incompatible species, but resource limitation might not let them take advantage of it. Thus, constraints from specific abiotic conditions could theoretically explain the similarity between self-compatible and self-incompatible species [17]. Furthermore, comparisons of pollen limitation between phenotypically specialized and generalized flowers reported ambiguous results. Larson and Barrett [11] found that species with specialized floral morphology and less accessible nectar did not differ from those with generalized morphology in the level of pollen limitation. Contrarily, Lázaro et al. [18] recorded that species with specialized flowers were more pollen limited than those with generalized flowers. Therefore, individual floral traits can explain only a small part of variation in pollen limitation [11].

New insights into the variation of pollen limitation causes could be provided by exploration of correlative effects between multiple reproductive and functional traits and pollen. For example, even key traits like dichogamy or clonality have not been thoroughly explored in this context. While dichogamy level has been suggested as ensuring higher autonomous seed set in plants exposed to outcross pollen limitation [19], clonality may provide reproductive advantage for obligate outcrossing species that are in the higher risk of pollen limitation [20].

In this study, we applied pollen supplementation to evaluate the level of pollen limitation in a community of flowering plant species in a wet meadow in a fragmented cultural landscape in Central Europe. Consequently, we correlated the pollen limitation with multiple functional traits of the plant species. We hypothesized that the degree of pollen limitation of plant species will be influenced by (i) a type of breeding system, (ii) floral traits important for pollinator attraction and foraging technique, and (iii) their degree of functional specialization on pollinators. We expected that plants visited by a broad spectrum of different insect functional groups (i.e., bees, flies, beetles, etc.) will be less pollen limited. We also predicted that the lower pollen limitation would occur in pollinator-attractive plants with abundant nectar rewards and/or more open flowers. Last but not the least, we provided a comprehensive pollen limitation dataset from Central Europe, a region previously largely neglected in pollination networks and pollen limitation studies [13].

2. Results

2.1. Seed Production and Seed Weight

The differences in both seed production and seed weight between pollen-supplemented and naturally pollinated flowers at the community level were statistically significant (permutational MANOVA; Pseudo-F = 3.99, $p = 0.023$, and Pseudo-F = 6.92, $p = 0.005$, respectively). At the species level, we found a statistically significant positive increase in seed production after pollen supplementation in seven species (i.e., Table 1): *Anemone nemorosa*, *Lysimachia vulgaris*, *Lychnis flos-cuculi*, *Potentilla palustris*, *Aegopodium podagraria*, *Ranunculus auricomus*, and *Stellaria graminea*. The mean weight per seed of the pollen-supplemented flowers was significantly higher in two species, *Lychnis flos-cuculi* and *Cardamine pratensis*. The capsula weight after the pollen supplementation significantly increased in *Dactylorhiza majalis*. PLs for all individual species are presented in Figure 1.

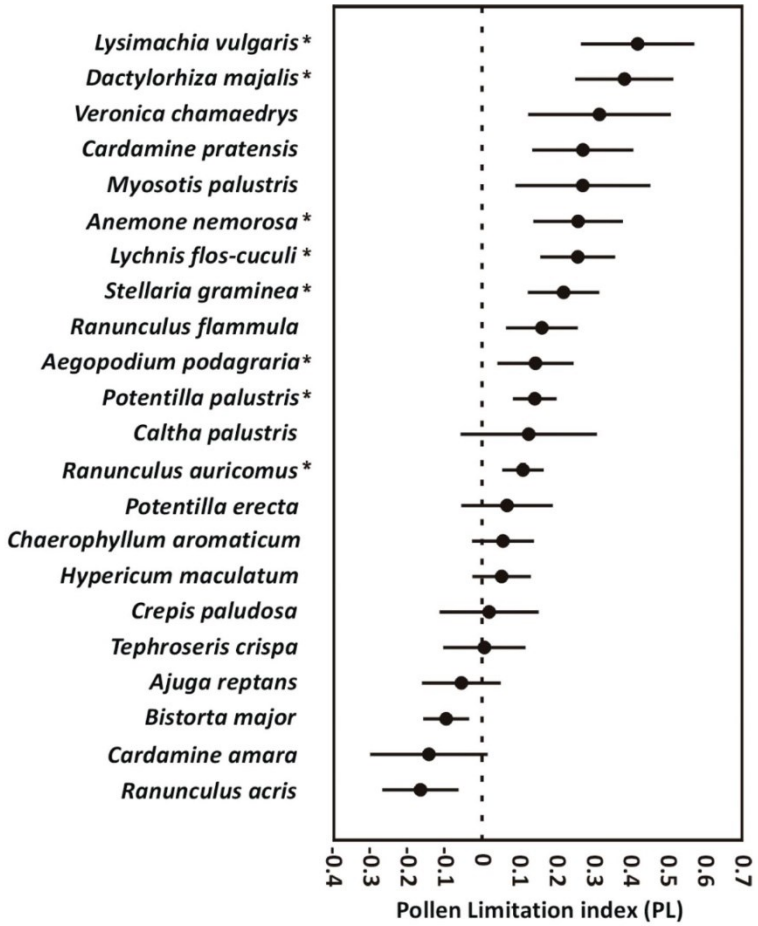

Figure 1. Pollen limitation index (PL) with standard error for 22 plant species from a wet meadow community in Central Europe. Asterisks denote statistically significant values of PL in individual plant species.

Table 1. Seed production and seed weight for the supplementary hand-pollinated and naturally-pollinated flowers. Asterisks denote p-values of species with statistically significant one-sided test in non-parametric permutation ANOVA. For detail descriptions of the different units used for each species, see section Methods and Materials.

	No. of Seeds						Weight of a Seed (μg)					
Family	**Control**		**Supplemented**		**PERMANOVA**		**Control**		**Supplemented**		**PERMANOVA**	
Species	**Mean**	**(std.dev)**	**Mean**	**(std.dev)**	**Pseudo F**	***p***	**Mean**	**(std.dev)**	**Mean**	**(std.dev)**	**Pseudo F**	***p***
Apiaceae												
Aegopodium podagraria	16.76	(±8.85)	21.00	(±8.91)	3.75	0.040 *	1.64	(±0.82)	1.95	(±0.90)	1.32	0.138
Chaerophyllum aromaticum	20.63	(±11.8)	20.74	(±9.3)	0.00	0.479	2.33	(±1.06)	2.45	(±0.92)	0.75	0.218
Asteraceae												
Crepis paludosa	30.07	(±13.56)	30.20	(±10.77)	0.00	0.487	0.37	(±0.21)	0.34	(±0.17)	0.15	0.363
Tephroseris crispa	55.95	(±30.02)	52.60	(±16.07)	0.24	0.319	0.24	(±0.11)	0.26	(±0.11)	0.44	0.269
Boraginaceae												
Myosotis palustris	1.25	(±1.22)	1.83	(±1.34)	3.01	0.063	0.23	(±0.20)	0.26	(±0.17)	0.16	0.360
Brassicaceae												
Cardamine amara	9.72	(±7.05)	7.94	(±7.53)	0.68	0.208	0.05	(±0.03)	0.03	(±0.03)	2.46	0.065
Cardamine pratensis	5.89	(±5.2)	8.11	(±5.18)	2.53	0.066	0.15	(±0.09)	0.19	(±0.07)	4.90	0.021 *
Caryophyllaceae												
Lychnis flos-cuculi	76.32	(±55.12)	100.68	(±46.74)	5.10	0.021 *	0.08	(±0.06)	0.12	(±0.04)	8.43	0.005 *
Stellaria graminea	7.54	(±6.00)	9.57	(±4.86)	3.12	0.045 *	0.19	(±0.11)	0.22	(±0.06)	2.99	0.051
Hypericaceae												
Hypericum maculatum	339.10	(±142.53)	364.45	(±161.06)	1.03	0.165	0.04	(±0.01)	0.04	(±0.01)	0.44	0.259
Lamiaceae												
Ajuga reptans	3.40	(±1.12)	3.13	(±1.13)	0.52	0.261	0.99	(±0.38)	1.01	(±0.34)	0.02	0.431
Orchidaceae												
Dactylorhiza majalis *	0.005	(±0.00)	0.01	(±0.01)	10.15	0.001 *						
Plantaginaceae												
Veronica chamaedrys	2.00	(±2.95)	3.00	(±3.42)	0.68	0.208	0.08	(±0.10)	0.11	(±0.10)	0.46	0.252

Table 1. *Cont.*

	No. of Seeds						Weight of a Seed (µg)					
Family	**Control**		**Supplemented**		**PERMANOVA**		**Control**		**Supplemented**		**PERMANOVA**	
Species	**Mean**	**(std.dev)**	**Mean**	**(std.dev)**	**Pseudo F**	***p***	**Mean**	**(std.dev)**	**Mean**	**(std.dev)**	**Pseudo F**	***p***
Polygonaceae												
Bistorta major	79.50	(±45.13)	69.85	(±36.36)	2.16	0.080	5.21	(±1.50)	5.20	(±1.53)	0.00	0.477
Primulaceae												
Lysimachia vulgaris	12.65	(±16.24)	39.05	(±36.66)	9.29	0.003 *	0.22	(±0.15)	0.24	(±0.13)	0.35	0.281
Ranunculaceae												
Anemone nemorosa	7.11	(±4.69)	11.61	(±7.01)	10.71	0.002 *	2.13	(±0.99)	2.32	(±0.84)	0.33	0.283
Caltha palustris	30.82	(±39.43)	32.82	(±27.1)	0.03	0.429	0.43	(±0.23)	0.47	(±0.20)	0.31	0.308
Ranunculus acris	19.33	(±7.08)	15.44	(±8.12)	3.43	0.041	1.23	(±0.23)	1.20	(±0.45)	0.06	0.397
Ranunculus auricomus	10.40	(±3.28)	11.80	(±2.91)	3.64	0.041 *	2.01	(±0.48)	2.00	(±0.38)	0.01	0.460
Ranunculus flammula	31.89	(±16.94)	37.83	(±12.67)	2.23	0.076	0.34	(±0.15)	0.38	(±0.06)	0.75	0.213
Rosaceae												
Potentilla erecta	5.55	(±3.14)	6.15	(±3.34)	0.56	0.238	0.54	(±0.24)	0.52	(±0.24)	0.12	0.369
Potentilla palustris	219.11	(±80.41)	253.16	(±54.14)	4.05	0.029 *	0.26	(±0.08)	0.28	(±0.11)	0.37	0.276

* In *Dactylorhiza majalis* the capsule weight in grams was used as a proxy for the number of developed seeds.

2.2. Traits Correlations

Our tests revealed that PL was significantly related only to the number of pollinator functional groups (Table 2 and Figure 2). We found no significant relationship between PL and other tested traits, including the multiple regression with all traits ($F = 0.83$, $p = 0.57$). The only trait selected by the AIC-based stepwise selection was again the plant specialization. All correlation indices between particular quantitative floral traits are presented (Table S1).

Both models, the unimodal and the linear, were significant (unimodal: $F = 7.95$, $p = 0.003$, Figure 2B; linear: $F = 4.39$, $p = 0.049$, Figure 2A). However, because of the relatively small number of target plant species, this unimodal relationship may be greatly affected by outlying values at the edges.

Table 2. Linear regressions of selected traits with pollen limitation. Asterisks denote *p*-values of species with statistically significant test.

Traits	F-Statistic	DF	*p*-Value
Specialization (No. of pollinator functional groups)	4.39	20	0.049 *
Clonality	1.24	20	0.278
Dichogamy	0.30	20	0.586
Sugar content	1.29	20	0.268
No. of open flowers	0.66	20	0.426
Self-compatibility	0.70	20	0.411
Autonomous selfing	0.17	20	0.681

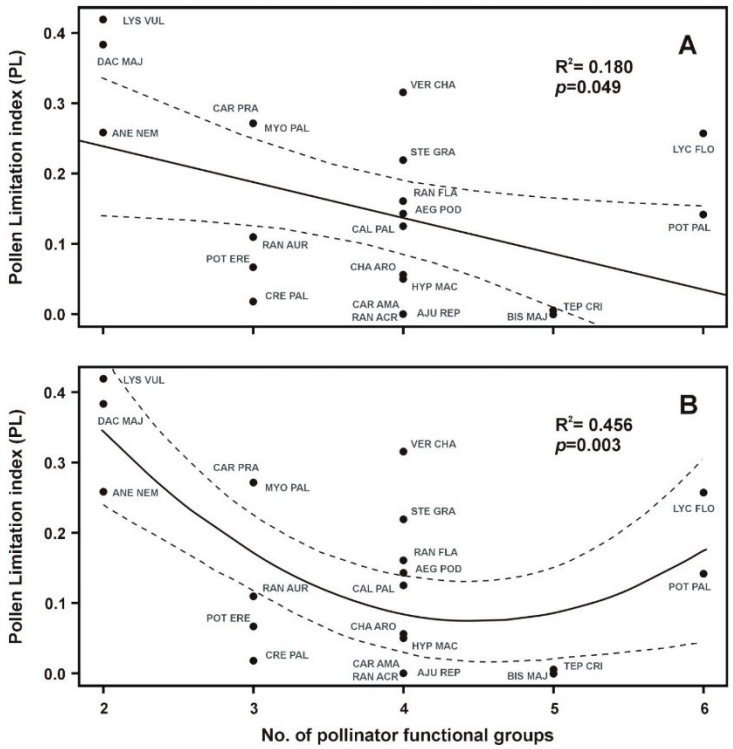

Figure 2. Linear (**A**) and polynomial (**B**) regression of pollen limitation index (PL) with plant specialization (number of functional groups of flower visitors). Dashed lines denote the 95% confidence interval for the model curve. Plant species (see complete list in Table 1) are presented as three letter abbreviations.

3. Discussion

Pollen limitation is generally considered a common phenomenon and many comparative studies report relatively high occurrence (62–73%) in various habitats [1,10]. However, we only recorded significant pollen limitation for approximately 41% of species (9 out of 22 surveyed) in our wet meadow community. Our findings are in concordance with a similar unusually low occurrence of pollen limitation in a temperate grassland community in western Norway [21]. This study focused on pollen limitation and its relationship to plant species visitation rates and specialization levels and revealed only two out of eleven (~18%) studied plant species to be significantly pollen limited. Moreover, Bennett et al. [13] even documented no pollen limitation in investigated study of nine species in a Romanian meadow community. It might seem that the low levels of pollen limitation revealed in the pollen supplementation experiments are in agreement with the assumptions from the model by Haig and Westoby [7], which stipulates that seed set in flowering plants should be equally limited by both pollen and resource availability. It further suggests that pollen supplementation should not increase seed set in populations at their evolutionary equilibrium, because resources should be unavailable for maturation of their additional fertilized ovules. However, Burd [22] adjusted this model for stochastic variation in both ovule fertilization and resource availability, which made the model broadly in accordance with the recent meta-analysis [1,10], in which pollen limitation is found in most surveyed species.

The reported inconsistencies in the magnitude of pollen limitation could stem from several non-mutually exclusive reasons:

(1) *Effect of sampling size and experimental design.* Using power tests (via simulation) for pollen supplementation experiments, Thomson [23] illustrated that moderate pollination deficits of up to 15% will usually not be detected with sample sizes of 20 individuals, and even 40 are insufficient for minor deficits. But, unfortunately, lower sampling effort (such as 20–30 individuals in our study) is an inevitable result of various logistic constrains and trade-offs between the data quantity and quality in most community studies [13,18,21,24].

(2) *Publication bias.* The community approach, where multiple plant species are studied simultaneously, may lead to a better understanding of patterns in pollen limitation. It is because environmental characteristics, such as nutrient levels within a given community, are relatively homogenous in such studies and the role of plant traits in pollen limitation can, therefore, be better assessed. Nevertheless, there have been few studies focused on the relationships between plant traits and pollen limitation across whole communities [18,21,24–26]. All these studies recorded lower levels of pollen limitation in natural systems compared to the pollen limitation documented in comprehensive reviews that are mostly based on single-species studies [1,10]. Therefore, the publication bias, favoring statistically significant responses which then become available for further studies, together with the omission of "grey literature" and studies not written in English [27], complicates our understanding of pollen limitation [14].

(3) *Effect of pollinator abundance.* Hegland and Totland [21] discussed their results of low pollen limitation in the context of a possible higher pollinator abundance in the studied community, which could substantially reduce the quantitative pollen limitation. A partial cause of low pollen limitation in our study could be that the targeted semi-natural locality is situated in a relatively well-preserved and mosaic-like landscape with a limited influence of intensive agriculture. Such semi-natural, diverse, and heterogeneous environments support pollination services [28,29] and thus increase the plant reproductive success, as suggested by Bennett et al. [13] in their Romanian meadow community.

(4) *Effect of plant community composition and study species selection.* In our investigated community, only a few plant species with morphologically highly specialized flowers, which are expected to be more prone to pollen limitation, were present. Therefore, this community may have a lower pollen limitation than communities with a greater proportion of specialized flowers.

(5) *Choice of the pollen limitation measure*. An important factor determining the recorded magnitude of pollen limitation may also be the choice of its measure. Knight et al. [14] compared 263 studies working with different measurements of the production component of reproduction and revealed the largest effect for relative fruit set, and the lowest effect for production of seeds/flower and seeds/fruit. However, because the magnitude of pollen limitation was inter-correlated among these response variables, Knight et al. [14] assumed that pollen limitation occurs simultaneously at different stages of the plant reproduction, but with varying intensity. Also in our study the numbers of pollen-limited species varied substantially between the two applied measures, seed production and seed mass. Furthermore, Hegland and Totland [21] pointed out that the two main components of plant reproductive success, seed production and seed mass, are often not included in the same studies.

In our studied community, two species showed significant positive seed weight response after pollen supplementation, though we expected the negative relationship. Several studies demonstrated that seed mass decreases with pollen availability because of seed size–number trade-off [1]. For example, Ågren et al. [30] recorded reduced mean seed size in hand-supplemented *Primula farinosa* by about 12%, but a larger total mass of seeds than in naturally pollinated plants. The opposite effect, i.e., increased seed weight after pollen supplementation could be explained by the increased pollen quality [21]. Aizen and Harder [31] suggested that the cross-pollen used for supplementation may have higher quality than the mixture of self- and cross-pollen available under the natural pollination. It seems evident that the magnitude of pollen limitation is dependent on the treatment level, e.g., whether the experimental design is applied only on a fraction of the plant's flowers or on the whole plant [32]. Unfortunately, because we treated only flower pairs, we can only speculate on the proportion of resource allocation in our study [33]. The low differences in the seed weight between the treatments could be caused by the ability of the plant to compensate for any possible higher cost of an additional seed production induced by the supplemental pollination in only one flower.

Species with high PL values have specific ecological features. *Lysimachia vulgaris* is pollinated by highly specialized oil-collecting *Macropis* bees [34]. The species is also dominant in the locality, producing many flowers at same time, which suffer from competition for pollinators. An orchid *Dactylorhiza majalis* offers no reward to pollinators, which are deceived by showy flowers. Deceptive orchids typically produce little fruits [35]. *Anemone nemorosa* is flowering very early in the vegetation season when visitors are limited by unexpected weather conditions, especially by low temperature [36]. Species with low PL mainly belong to a group of plants with many, generalized flowers with easily available nectar rewards. But nectar is not the only reward which is offered by plants. Pollen is also a very important attractant for visitors. Unfortunately, we do not have adequate data about pollen production for the investigated plant species. However, the main pollinator group recorded on the studied locality appears to be hoverflies, feeding on both nectar and pollen. A wide range of hoverfly larvae are associated with accumulations of wet, rotting vegetation in ponds and ditches, which are common nearby. The other abundant groups were other flies and honey bees).

Despite analyses of several floral and life history traits connected to plant reproduction, we only found the significant relationship of PL to the number of pollinator functional groups. This finding is in accordance with the meta-analysis of pollen limitation in different world regions [16,37,38], where the more pollinator-specialized plant species were also more pollen limited (but see [21]). However, Lázaro et al. [18] pointed out that this relationship is not entirely clear and it is very important to distinguish between morphological (based on floral shape) and ecological (based on realized interactions) specialization. They found a strong negative relationship between pollen limitation and ecological generalization, but only for species with the morphologically specialized flowers. As a possible explanation they suggested that the morphologically specialized flowers benefit more from generalizing their pollination system in the lack of a primary pollinator [18]. The high ecological generalization may however result in the stronger pollen limitation because of lower flower-visitor diversity with abundant low-efficiency pollinators transporting high loads of incompatible pollen [39].

Accordingly, we recorded stronger pollen limitation in the species with specialized, as well as highly generalized pollination systems. This supports the prediction that many mutual relationships between plants and visitors should be non-linear [40,41].

However, assuming the validity of the linear model, we expect higher diversity in conspecific pollen load with increasing number of pollinator functional groups because of pollen grains coming from a wider range of donors. It could stimulate pollen competition and successful pollination. On the other hand, assuming the validity of the unimodal model which has much higher significance value than the linear model, increasing the number of pollinator functional groups may involve less-specific pollinators. These may clog stigmas with higher loads of heterospecific pollen, which could decrease the reproductive success of plants. Nevertheless, we can only speculate about the accuracy of using one model, because a detailed study on pollinator group effectiveness for each particular plant species would be necessary to make an unambiguous conclusion.

Our results describe pollen limitation based on observation from a single season. As seasonal course of climate affects both plant phenology and insect activity, thus we cannot exclude that pollen limitation will be different in other seasons.

4. Materials and Methods

4.1. Study Site

Our focal plant community was situated in a semi-natural wet meadow near the Chobotovský rybník pond in the landscape protected area of Železné hory (Bohemian-Moravian Highlands, Czech Republic; 535 m a.s.l., 49°46′57″ N, 15°50′17″ E). Mean annual temperature is 6.4 °C and annual precipitation is 745 mm. The subsoil is formed by fluvial sandy loam and sandy gravels. The meadow, with an area of 1.2 ha, is isolated from the surrounding agricultural landscape by a high forest. The meadow is mowed once a year. By phytosociological classification [42], the meadow belongs to the alliance *Calthion palustris* with vegetation dominated by *Agrostis canina*, *Scirpus sylvaticus*, *Lysimachia vulgaris*, and *Filipendula ulmaria*. We collected data on the 22 most abundant insect-pollinated plant species flowering between the beginning of May and mid-July 2017. During the vegetation season, 51 insect-pollinated species were flowering.

4.2. Pollen Limitation

Supplemental hand-pollination was applied on randomly selected individuals (20–30 per species) with at least two open flowers in similar phenological phases. One flower was supplemented by conspecific pollen from plants minimally two meters apart to reduce the genetic closeness and the second was left to natural pollination as a control. Both hand-pollinated and control flowers were marked by colored cotton yarn loosely knotted under the flowers. In species producing just a single flower (*Anemone nemorosa*) or compact inflorescences (*Bistorta major*) on a single shoot, we applied the treatments on two neighboring individuals. In Asteraceae species (*Crepis paludosa* and *Tephroseris crispa*) the treatments were applied on two whole capitula, and in Apiaceae species with compound umbels (*Aegopodium podagraria* and *Chaerophyllum aromaticum*) the treatments were applied on two umbellets from two different umbels. All species with flowers in compact and sequentially opening inflorescences (i.e., *Aegopodium podagraria*, *Bistorta major*, *Chaerophyllum aromaticum*, *Crepis paludosa* and *Tephroseris crispa*) were hand-pollinated repeatedly for several consecutive days throughout the whole flowering period. We collected anthers with visible pollen grains (verified by hand lens) and rubbed them over the receptive stigmas (successful deposition was again verified by hand lens). For compact inflorescences, capitulas, or umbels, we rubbed the whole donor unit over the recipient one. After the marked flowers wilted, their maturing ovaries were enclosed in fine nylon mesh bags to avoid any seed loss. We counted all viable seeds from the collected flowers or inflorescences and measured mean weight per seed. Because *Dactylorhiza majalis* produced numerous very small seeds, we used the capsule weight as a proxy for the number of developed seeds.

4.3. Plants Traits

We collated information on seven plant characteristics: Type of breeding system (i.e., extent of self-compatibility and autonomous selfing), level of dichogamy, clonality, amount of nectar reward, number of open flowers, and plant specialization on pollinator functional groups. Data on the extent of self-compatibility and autonomous selfing for 18 of the target species were obtained from our greenhouse pollination experiment [43]. Data on dichogamy were extracted from the Biolflor database [44] and transformed from the original seven categories to a continuous variable ranging from 0 to 0.5, with the value 0.5 denoting an absence of dichogamy (i.e., simultaneous presence of male and female organs). The missing data on breeding type (four cases) and level of dichogamy (two cases) were replaced by average values from the whole dataset. The clonal multiplication (i.e., number of vegetative offspring per maternal shoot per year) was extracted from the Clo-Pla database [45]. The daily sugar production in nectar reward was determined in the field for 15 flowers per plant species. Flowers from different plant specimens were bagged at their full anthesis for 24 h after which nectar was extracted. Nectar was washed with distilled water using a 100-µL Hamilton syringe and stored in a refrigerator prior to freezing, following Morrant et al. [46]. The amount of nectar sugars was quantified by high-performance liquid chromatography (HPLC) using the ICS-3000 system (Dionex), with an electrochemical detector and CarboPac PA 1 column. The nectar production was expressed in milligrams of nectar sugars per flower/day (Table 3). Mean number of open flowers per species was calculated from 60 specimens per species from three meadows in the study region.

Table 3. The daily nectar sugars production of plant species per flower.

Family	**Nectar Sugars (mg)**		
Species	**Flower/Day**		**std.dev**
Apiaceae			
Aegopodium podagraria	0.0149	±	0.007
Chaerophyllum aromaticum	0.0167	±	0.0124
Asteraceae			
Crepis paludosa	0.0099	±	0.0123
Tephroseris crispa	0.0216	±	0.0269
Boraginaceae			
Myosotis palustris	0.0039	±	0.0076
Brassicaceae			
Cardamine amara	0.0184	±	0.0196
Cardamine pratensis	0.0193	±	0.0343
Caryophyllaceae			
Lychnis flos-cuculi	0.2666	±	0.1266
Stellaria graminea	0.1185	±	0.0594
Hypericaceae			
Hypericum maculatum	0.0005	±	0.0003
Lamiaceae			
Ajuga reptans	0.2150	±	0.0671
Orchidaceae			
Dactylorhiza majalis	0.0014	±	0.0011
Plantaginaceae			
Veronica chamaedrys	0.1540	±	0.0548

Table 3. *Cont.*

Family	Nectar Sugars (mg)		
Species	Flower/Day		std.dev
Polygonaceae			
Bistorta major	0.0598	±	0.0152
Primulaceae			
Lysimachia vulgaris	0.0009	±	0.0017
Ranunculaceae			
Anemone nemorosa	0.0004	±	0.0004
Caltha palustris	0.0006	±	0
Ranunculus acris	0.0526	±	0.0375
Ranunculus auricomus	0.0144	±	0.0074
Ranunculus flamula	0.0314	±	0.0175
Rosaceae			
Potentilla erecta	0.0804	±	0.0722
Potentilla palustris	3.2997	±	1.0182

Plant functional specialization was expressed as the number of pollinator functional groups that touched anthers and/or stigmas during foraging. The pollinator spectrum for each plant species was counted from videos recorded in the field using portable video systems of VIVOTEK (IB8367-T) and MILESIGHT (MS-C2962-FPB-IR60m) cameras. In total, 72 h (equally covering day and night) per plant species were recorded in three different localities in the vicinity of the study area. All pollinators were split into eleven functional groups: ants, beetles, bumblebees, butterflies, honeybees, hoverflies, long-tonged flies, moths, other bees, other flies, and other hymenopterans (Table 4). Groups which were represented by fewer than three visitors per plant species were excluded from the analyses to avoid random visits.

Table 4. Recorded pollinator spectrum of plant species (total number of visits per 72 h of video recording).

Family / *Species*	Ants	Beetles	Bumblebees	Butterflies	Honey Bees	Hoverflies	Long-Tonged Flies	Moths	Other Bees	Other Flies	Other Hymenopterans	Total Visits by Plants
Apiaceae												
Aegopodium podagraria	1	49			2	4		2	13	52		123
Asteraceae												
Crepis paludosa		1		1	25	8			15	1	1	52
Tephroseris crispa		3	5	2	1	32			4	19		66
Boraginaceae												
Myosotis palustris		9				12				29		50
Brassicaceae												
Cardamine amara		9				12				29		50
Cardamine pratensis		2			1	9		1	3	45		61
Caryophyllaceae												
Lychnis flos-cuculi	3	1		47	76	11		8	4	2		152
Stellaria graminea	1	5		1	1	53			5	31		97
Hypericaceae												
Hypericum maculatum		1	7	2	129	55		1		4		199
Lamiaceae												
Ajuga reptans	4		22	3		7			1	1		38
Orchidaceae												
Dactylorhiza majalis	1	1	1	3	1		1	3				11
Plantaginaceae												
Veronica chamaedrys		3			1	7			5	11		27
Polygonaceae												
Bistorta major		5	1	1	21	4			3	7		42
Primulaceae												
Lysimachia vulgaris		1				11			12			24
Ranunculaceae												
Anemone nemorosa		2			1	4			1	5		13
Caltha palustris	1			1	15	77			3	41		138
Ranunculus acris		7	1	1	1	20		1	10	20		61
Ranunculus auricomus	1	5				20			1	5		32
Ranunculus flamula		16				19			5	16		56
Rosaceae												
Potentilla erecta					1	94			7	9	2	113
Potentilla palustris		2	9	7	3	52		1	9	18		101
Total visits by groups	12	122	46	69	279	511	1	17	101	345	3	

4.4. Data Analysis

Differences in reproductive success between supplemental hand-pollination and natural pollination at community and species levels were tested for both seed production and seed weight. Because the data contained many zero values and even after transformation did not meet the normality assumption, we applied non-parametric tests. At community level we used permutational MANOVA with permutation of residuals under a reduced model, where treatment served as fixed and plant species as random factor. At species level we used a one-sided test in non-parametric permutational ANOVA. Both tests were done within the PERMANOVA package in Primer 6 software [47].

We calculated the pollen limitation index (PL) as $PL = (P_s - P_o)/P_{max}$ (P_s or P_o) [48], where P_s is the number of seeds from pollen-supplemented flowers, P_o is the number of seeds from open-pollinated flowers, and P_{max} is the larger of the two values (P_s or P_o). For all subsequent analyses, similarly to Larson and Barrett [11], we established zero as the lower boundary of the PL, because any negative indices likely resulted from a potential experimental error [49], and therefore are not meaningful in the context of our study.

Although most studies assumed a linear relationship between possible plant seed set and traits, some studies predicted numerous relationships between plant and visitors to be non-linear [41,50]. Thus, all correlations of PL with plant characteristics were tested using both simple and multiple linear as well as unimodal regressions. Because of right-skewed distribution, the values for nectar production and number of flowers were log-transformed prior to analysis. For selection of the best model we used AIC stepwise selection. All analyses, unless otherwise specified, were conducted using R [51].

5. Conclusions

Our study recorded significant pollen limitation for approximately a third of species occurring in a wet meadow community. It was much lower than what has been reported in the previous reviews of single species studies, but higher when compared with all other community level studies. The discrepancy in the results of these studies can be attributed to several issues, such as sampling and publication biases. Except for the number of pollinator functional groups, we could not attribute pollen limitation to the other measured floral and life history traits. Therefore, some additional traits may also be contributing to patterns of pollen limitation. Such additional traits could be extrinsic traits (e.g., regional plant diversity) because interactions between extrinsic and floral or life history traits may be the major driver of pollen limitation in communities [52]. Finally, other overlooked and possibly important factors can be spatial and temporal variations in pollen limitation within and among communities.

Supplementary Materials: The following are available online at http://www.mdpi.com/2223-7747/9/5/640/s1, Table S1: Correlation matrix of selected traits.

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Supplementary material

Supplementary Table 1. Correlation matrix of selected traits.

	Specialization	Clonality	Dichogamy	Sugar content	No. of open flowers	Self-compatibility	Autonomous selfing
Specialization							
Clonality	-0.29						
Dichogamy	**-0.47**	**0.43**					
Sugar content	**0.68**	-0.36	-0.40				
No. of open flowers	0.01	0.01	-0.30	-0.14			
Self-compatibility	0.30	-0.07	-0.27	0.31	-0.04		
Autonomous selfing	-0.08	-0.21	-0.02	-0.09	-0.12	**0.50**	

CHAPTER VI

Klomberg Y, Dywou Kouede R, Bartoš M, Mertens JEJ, Tropek R, Fokam EB, Janeček Š (2019). The role of ultraviolet reflectance and pattern in the pollination system of Hypoxis camerooniana (Hypoxidaceae). *AoB PLANTS* 11(5): plz057. (IF_{2019}: 2.238).

YK helped design the experiment and was involved in the data collection and processing. He also participated in performing of additional analyses requested during the review process and their interpretations, and led writing of the manuscript, under supervision of ŠJ.

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STUDIES

The role of ultraviolet reflectance and pattern in the pollination system of *Hypoxis camerooniana* (Hypoxidaceae)

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Abstract

Apart from floral morphology and colours perceived by the human eye, ultraviolet (UV) reflectance acts as an important visual advertisement of numerous flowering plant species for pollinators. However, the effect of UV signalling on attracting pollinators of particular plant species is still insufficiently studied, especially in the Afrotropics. Therefore, we studied the pollination system of *Hypoxis camerooniana* in montane grasslands of Mount Cameroon, West/Central Africa. We focused mainly on the effects of the flowers' UV reflectance on its visitors. We experimentally removed UV reflection from petals either completely or partially. Thereafter, flower visitors were recorded and pistils were collected post-flowering to quantify germinated pollen tubes per treatments. The most important visitors were bees, followed by flies. Due to their contacts with reproductive organs bees are considered as the primary pollinators. Visitation rates were lower when UV reflectance was completely removed, whereas the decrease of frequency on half-treated flowers did not differ significantly from control treatments. The complete removal of UV also affected bees' landing behaviour, but not that of flies. We showed that the presence of UV reflectance is more important than UV pattern for bees visiting flowers of *H. camerooniana*. We hypothesize that exploiting all flowers irrespective of their pattern can be more efficient for pollinators in the open grasslands of high altitudes to spot these relatively scarce flowers by their UV reflectance. Furthermore, we highlight the necessity of both experimental and natural controls in similar studies to control for additional effects of the used UV manipulations.

Keywords: Afromontane grasslands; floral traits; foraging behaviour; Mount Cameroon National Park; pollination interactions; UV manipulation.

Introduction

Unlike humans, many insect pollinators are sensitive to the ultraviolet (UV) part of the electromagnetic light spectrum in addition to the visible spectrum (Briscoe and Chittka 2001). Ultraviolet light is reflected by flowers of ~25 % of angiosperms, with the highest reflectance found in plant species with yellow flowers (Chittka *et al.* 1994; Papiorek *et al.* 2016). Consequently,

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the UV vision helps floral visitors in recognition of individual flowers of such plants which differ in their UV colouration from other plants in the community (Johnson and Andersson 2002). To increase distinction by certain groups of pollinators, some flowers create a contrasting pattern of UV absorbance and reflectance on the surface of their petals, whereas others contrast petals and reproductive parts by an inverse pattern of absorbance and reflectance of UV light. Floral guides (Penny 1983; Dinkel and Lunau 2001; Lunau 2006; Papiorek *et al.* 2016) and the so-called bullseye patterns (Lunau 1992a; Koski and Ashman 2014, 2015a, b), which has reflecting apices and absorbing bases of petals, are among the most commonly known examples of this phenomenon. These UV patterns are believed to improve the identification of the landing and/or foraging parts of flowers, or mimic such parts to the pollinator (Lunau *et al.* 2017). Their importance was shown in numerous studies revealing the influence of UV patterns on pollinator visitation preferences (e.g. Burr *et al.* 1995; Campbell *et al.* 2010; Horth *et al.* 2014; Koski and Ashman 2014; Peterson *et al.* 2015) and behaviour (Hansen *et al.* 2012).

The specific colour vision, which includes UV, of some insects and spectral properties of flowers have evolved into mutualistic relationships between plants and their pollinators. One of the best understood systems of vision is that of bees (Dyer *et al.* 2015). von Helversen (1972) measured the capability of honeybees to distinguish colours and showed that bees best discriminate wavelengths at ~400 and 500 nm where the spectral sensitivity curves of UV, blue and green photoreceptors overlap. Peitsch *et al.* (1992) quantified this further, showing the trichromatic vision of 43 different taxa of hymenopterans with the sensitivity peak of UV light at 340 nm, blue light at 430 nm and yellow light at 535 nm. Some specific colour patterns of flowers, such as floral guides, are of such importance for bee flower recognition that they were included into *melittophily*, i.e. the pollination syndrome related to bees (Faegri and van der Pijl 1979; Lunau 1992a; Fenster *et al.* 2004; Willmer 2011). However, the utility of these syndromes has been questioned recently (Ollerton *et al.* 2009), and some authors started using more precise systems, such as looking at single explanatory traits (e.g. Stang *et al.* 2009) or buzz pollination (e.g. De Luca and Vallejo-Marín 2013). The importance of UV patterns for bees was confirmed by a decreased frequency of flower visitation by different bee species after elimination of the UV reflection from petals of various plant species (Johnson and Andersson 2002; Peter and Johnson 2008; Welsford and Johnson 2012; Rae and Vamosi 2013; Brock *et al.* 2016).

Nevertheless, floral colour evolution has been influenced by numerous other factors (Grimaldi 1999; Friis *et al.* 2011; Song *et al.* 2015), such as floral defence against solar radiation (Robberecht and Caldwell 1978; Koski and Ashman 2015a). It was shown that the absorbance of UV-A by plant tissues can be related to plant protection against harmful UV-B radiation (Robberecht and Caldwell 1978; Caldwell *et al.* 1983). The importance of UV colour reflectance and absorbance can be manifested along the gradient of UV irradiation, e.g. towards high altitudes and the equator (Johnson *et al.* 1976; Beckmann *et al.* 2014; Koski and Ashman 2015a). It was shown that UV irradiance as the selection agent affects the size of the UV-absorbing floral centre (bullseye), with increases towards the equatorial ecosystems and along altitudinal gradients as well (Koski and Ashman 2015a, b). Despite long-term research on floral UV signalling many questions remain unanswered. For example, it is unclear if the ability of pollinators to recognize the flower is caused by any UV-reflecting area on the flower or if it is related to specific UV patterns. Additionally, we only have limited knowledge on how the common experimental manipulation using UV-absorbing creams (e.g. Johnson and Andersson 2002; Peter and Johnson 2008; Welsford and Johnson 2012; Rae and Vamosi 2013) generally affects the natural (i.e. unmanipulated) pollination system of the studied plant species.

To study the role of UV reflection in pollination we selected *Hypoxis camerooniana* (Hypoxidaceae) as model species. *Hypoxis camerooniana* is endemic to the mountains of south-western Nigeria and western Cameroon (Hutchinson *et al.* 1968) and therefore, better understanding its pollination system can help to better focus potential conservation efforts for both plant and its pollinators. Due to growing at low latitudes and high altitudes (above 2000 m a.s.l. on Mount Cameroon), *H. camerooniana* is exposed to intensive UV irradiance (Johnson *et al.* 1976; Beckmann *et al.* 2014). It has yellow UV-reflecting petals (Fig. 1) and yellow UV-absorbing anthers, consequently creating a contrasting central pattern in the flower. Firstly, we aimed to gain insights in the unknown pollination system of this endemic plant. Secondly, to study the role of UV colour on the visitation frequency, behaviour and pollination success of *H. camerooniana*, we used the same approach as Johnson and Andersson (2002) by manipulating flowers with an UV-absorbing cream either by complete removal of the UV reflectance, or by maintaining the UV reflectance on half of petals, i.e. changing the UV pattern (Fig. 1). Our study extends the previous work of, e.g., Johnson and Andersson (2002) by including a natural unmanipulated control to test the influence of the experimental treatments on the flower visitation frequency.

Materials and Methods

Study locality

This study was carried out at the montane grasslands above Mann's Spring (~2250 m a.s.l.) on Mount Cameroon, the highest mountain in western and central sub-Saharan Africa (4095 m a.s.l.; Cable and Cheek 1998). It is situated in the Southwest region of Cameroon (4.203°N and 9.170°E), offering a wide range of habitats (see Cable and Cheek 1998) and hosting a great biodiversity including endemics with exceptional ecological features (Bergl *et al.* 2007). Especially ecotones along the timberline, found also at Mann's Spring, harbour many species which are not found elsewhere on the mountain

Study plant

The genus *Hypoxis* contains an estimated 90 species in Africa, North and South America, South-Eastern Asia and Australia, with the centre of diversity in Southern Africa (Singh 1999). These plants are characterized by their bright yellow flowers, lanceolate and densely hairy leaves. They are weak competitors and thus grow mostly in places with low vegetation cover. *Hypoxis camerooniana* (Hypoxidaceae) is a perennial pyrophytic herb, restricted to high elevations of the Cameroonian Volcanic Line (Cable and Cheek 1998; GBIF Secretariat 2019). Its leaves are tristichous, 50 cm long and 0.5–2 cm wide, covered with golden hairs, recurved and ± prostrate to erect. On a scape up to 25 cm tall 5–7 flowers can be found (African Plant Database 2019). On Mount Cameroon, we always observed only 1 or 2 active flowers per plant. The flowers have a short lifespan, they open at daybreak and usually last 1 day or less.

Hypoxis species are used across Africa as traditional medicine and were reported to have a wide spectrum of pharmacological properties (Ncube *et al.* 2013). *Hypoxis hemerocallidea* has already

Figure 1. A flower of *H. camerooniana*: (A) a normal photograph, (B) a UV photograph of a non-manipulated flower, (C) a UV photograph with half absorbent cream treatment ($UV_{50\,\%}$), (D) a UV photograph with full absorbent cream ($UV_{100\,\%}$) treatment.

been known for its strong UV reflectance and due to its relatively robust flowers was used for manipulative experiments with UV-absorbing cream (Johnson and Andersson 2002). Outside of the two sweet-smelling species *H. fischerii* var. *zernyi* and *H. goetzei* East African *Hypoxis* flowers appeared to be without scent (Wiland-Szymańska 2009). So far, no nectar has been found in any *Hypoxis* species (Johnson and Andersson 2002; Rudall 2002; Ren *et al.* 2019).

Some studied *Hypoxis* species are pollinated by solitary bees and honeybees (Singh 1999; Johnson and Andersson 2002), while the autogamous *H. decumbens* attracts 'generalist insects, like dipterans' (Raimúndez and Ramirez 1998). Furthermore, pollen- and tepal-feeding beetles were observed in Southern and Eastern Africa (Steiner 1998; Wiland-Szymańska 2009). No data on pollination and visitation of *H. camerooniana* exist.

UV manipulation

The study was carried out in October and November 2016. To manipulate the UV patterns on flowers, we followed the approach of Johnson and Andersson (2002). The studied specimens of *H. camerooniana* were randomly selected in the study area. At daybreak, just after opening of selected buds, four different treatments were applied: (i) $UV_{100\,\%}$ *treatment*, i.e. complete removal of UV reflectance from the flower using UV cream on all petals of the flower; (ii) $FAT_{100\,\%}$ *treatment*, i.e. a control for $UV_{100\,\%}$, all petals were covered with duck preen gland fat, a non-UV-absorbing cream compound; (iii) $UV_{50\,\%}$ *treatment*, i.e. the UV cream was used on three out of six petals, covering every second petal; and (iv) $FAT_{50\,\%}$ *treatment*, i.e. a control for $UV_{50\,\%}$, every second petal was treated with the non-UV-absorbing compound. Both UV cream and non-UV-absorbing cream were applied carefully using cotton swabs. Besides these four treatments, natural (*Natural control*) non-manipulated flowers were studied to control for the effect of any treatment on flowers. The UV-absorbing chemicals were equal amounts of Parsol 1789® (butyl methoxydibenzoylmethane) and Parsol MCX® (ethylhexyl methoxycinnamate) dissolved in the duck preen gland fat as a solvent (at 40:60 w/w) by gentle heating (Andersson and Amundsen 1997; Johnson and Andersson 2002). On each day of the experiment, 10 plant specimens were selected in the grasslands and randomly treated, two replicates of each treatment per day, resulting in a total of 50 experimental plants. When two flowers were found on a single experimental plant, we applied the same treatment for both. Each experimental flower was recorded by a security camera (VIVOTEK IB8367-T with IR night vision) for 24 h following Mertens *et al.* (2018); however, most of the flowers were short-lived and closed at the beginning of the night after ~12 h of recording. Due to the short lifespan of flowers, all their visitors were certainly observed. Afterwards the recordings were watched, and all floral visitors

were noted. Besides arrival of visitors we also identified them to the most detailed taxon level as possible, and we noted their behaviour (both landing behaviour and activity after landing, e.g. feeding on pollen) and touches to reproductive organs, which allows us to better distinguish between visitors and potential pollinators.

After camera removal, stigmas of the recorded flowers were collected and stored in ethyl alcohol. Germinated pollen tubes were stained and counted later in the lab using fluorescence microscopy following the methods described by Dafni *et al.* (2005) to see how changes in visitation frequency potentially caused by the experimental treatment affect the plant's pollination success.

To check the floral UV pattern, UV photographs were taken using a Canon EOS 80D DSLR camera with a Helios 44-2 lens; UV conversion (i.e. replacing the internal hot mirror filter by a custom UV band pass filter) was done by LifePixel (Mukilteo, USA). During the picture taking, a 5-W UV flashlight was used for lighting (Fig. 1). To demonstrate the effect of experimental treatment, we measured reflectance of three flowers per treatment type ($FAT_{100\ \%}$, *Natural control* and $UV_{100\ \%}$) with 10 repeated measures per flower, using an Ocean Optics (Largo, USA) Jaz spectrometer. The graph depicts the mean of the repeated measures per treatment for the range of 300–700 nm (Fig. 2A). The bee and fly colour visual system was then mapped using the Troje model for flies (Troje 1993; Fig. 2B) and the colour hexagon for bees (Chittka 1992; Fig. 2C).

Statistical analyses

To standardize the sampling effort (differences in flower longevity, as well as in the case of one or two flowers per experimental plant), all visits were transformed to visitation frequencies (no. of visits per hour and flower). This visitation frequency data (no. of visits per hour and flower) did not show normal distribution due to an overabundance of null values. In consequence, we used non-parametrical tests, being a permutational analogue of ANOVA and MANOVA in PRIMER 6 v. 6.1.13 and Permanova+ v. 1.0.3 (Anderson *et al.* 2008). *Post hoc* tests were used to compare the frequencies between the different treatments, with the recording day treated as random effect. Similarly, the effects of treatments on insect behaviour were tested by a permutational MANOVA. To check differences in amount of morphospecies and pollen tubes (i.e. the non-frequency data) we tried to implement generalized mixed-effect models, specifically Poisson, quasi-Poisson and zero-inflated distributions. However, due to a combination of the high overabundance of zero values and the negative values of the maximum likelihood estimations of the models, we were not able to apply these parametric methods and therefore, the non-parametrical tests (permutational analogue of ANOVA) were applied as well. The dependency of the number of pollen tubes on visitation frequency was tested by linear regression in STATISTICA (Statsoft, Inc 2011).

Results

Visitors of *H. camerooniana*

Considering all 50 observed plants, a total of 281 visitors were recorded. During daytime the flowers were mostly visited by bees (192 visits) and flies (59 visits), the only other considerable group of visitors were skipper butterflies (four visits). All other visitors (five visits) were evaluated as accidental and thus merged (Fig. 3). All bee visitors were composed of a single abundant morphospecies of solitary bee (187 visits) and the substantially rarer honeybee (*Apis mellifera*; five visits). The less abundant flies were considerably more taxonomically diverse, compared to bees, with nine recognized morphospecies. Bees visited the studied flowers mainly during morning hours, whereas fly visitation was distributed throughout the day **[see Supporting Information—Figs S2 and S3]**. Contrastingly, night visitors were rare (21 visits by 10 morphospecies) and consequently, with much lower visitation frequencies **[see Supporting Information—Fig. S1]**. Based on contacts with reproductive organs (Fig. 3; Table 1) bees may be considered as the main pollinator.

Effects of UV pattern on visitors

Individual treatments significantly affected visitation frequency during the day (F_{ps} = 6.71; df = 4; P_{perm} < 0.001). $UV_{100\ \%}$ was significantly lower than all other treatments except $UV_{50\ \%}$, which differed from $FAT_{50\ \%}$ and *Natural control*. The highest visitation frequency was observed on untreated plants, but these did not significantly differ from the other two control treatments ($FAT_{100\ \%}$, $FAT_{50\ \%}$; Fig. 4). During the night, there was no significant treatment effect on frequency of flower visitors (F_{ps} = 0.36; df = 4; P_{perm} = 0.851). Visitation frequency was significantly affected by the treatment for both bees (F_{ps} = 6.13; df = 4; P_{perm} < 0.001) and flies (F_{ps} = 3.92; df = 4; P_{perm} = 0.009). In both visiting groups, $FAT_{100\ \%}$ and $FAT_{50\ \%}$ treatment has a significantly higher frequency than $UV_{100\ \%}$, but $UV_{50\ \%}$ was significantly lower than $FAT_{50\ \%}$ for flies only (Fig. 4). The non-treated control (*Natural control*) significantly differed from UV-manipulated plants for bees only (Fig. 4). There was no significant effect of treatment on the number of morphospecies observed on the flowers (F_{ps} = 2.08; df = 4; P_{perm} = 0.103).

Effects of treatments on visitor behaviour

We found a significant effect of treatment on bee landing behaviour (F_{ps} = 5.04; df = 4; P_{perm} = 0.004), but not on fly landing (F_{ps} = 1.08; df = 4; P_{perm} = 0.373). On $UV_{100\ \%}$-treated flowers, bees landed mostly on anthers, whereas in other treatments bees usually landed on the petals before moving to anthers and stigma (Fig. 5). When collecting pollen, bees usually touched both anthers and stigmas, whereas flies had considerably fewer contacts with the reproductive organs during their visits (Table 1). There was no significant effect of treatment on bees (F_{ps} = 0.49; df = 4; P_{perm} = 0.770) and flies (F_{ps} = 0.44; df = 4; P_{perm} = 0.903) behaviour after landing. Bees spent 95 % of the flower visit duration by collecting pollen, while flies spent most time (68 %) crawling, sitting and flying between individual floral parts **[see Supporting Information—Fig. S4]**.

Effect of treatment on the plant

The number of germinated pollen tubes significantly differed among treatments (F_{ps} = 3.66; df = 4; P_{perm} = 0.010), mainly due to a significantly higher number of pollen tubes germinated in non-manipulated flowers (Fig. 6). The pollen tube count increases with number of visits by bees (r = 0.57, P < 0.001; Fig. 7), but not of flies (r = –0.0073, P = 0.962).

Discussion

Our study demonstrated that *H. camerooniana* is mainly pollinated by bees, confirming previous studies on pollination of *Hypoxis* plants (Singh 1999; Johnson and Andersson 2002),

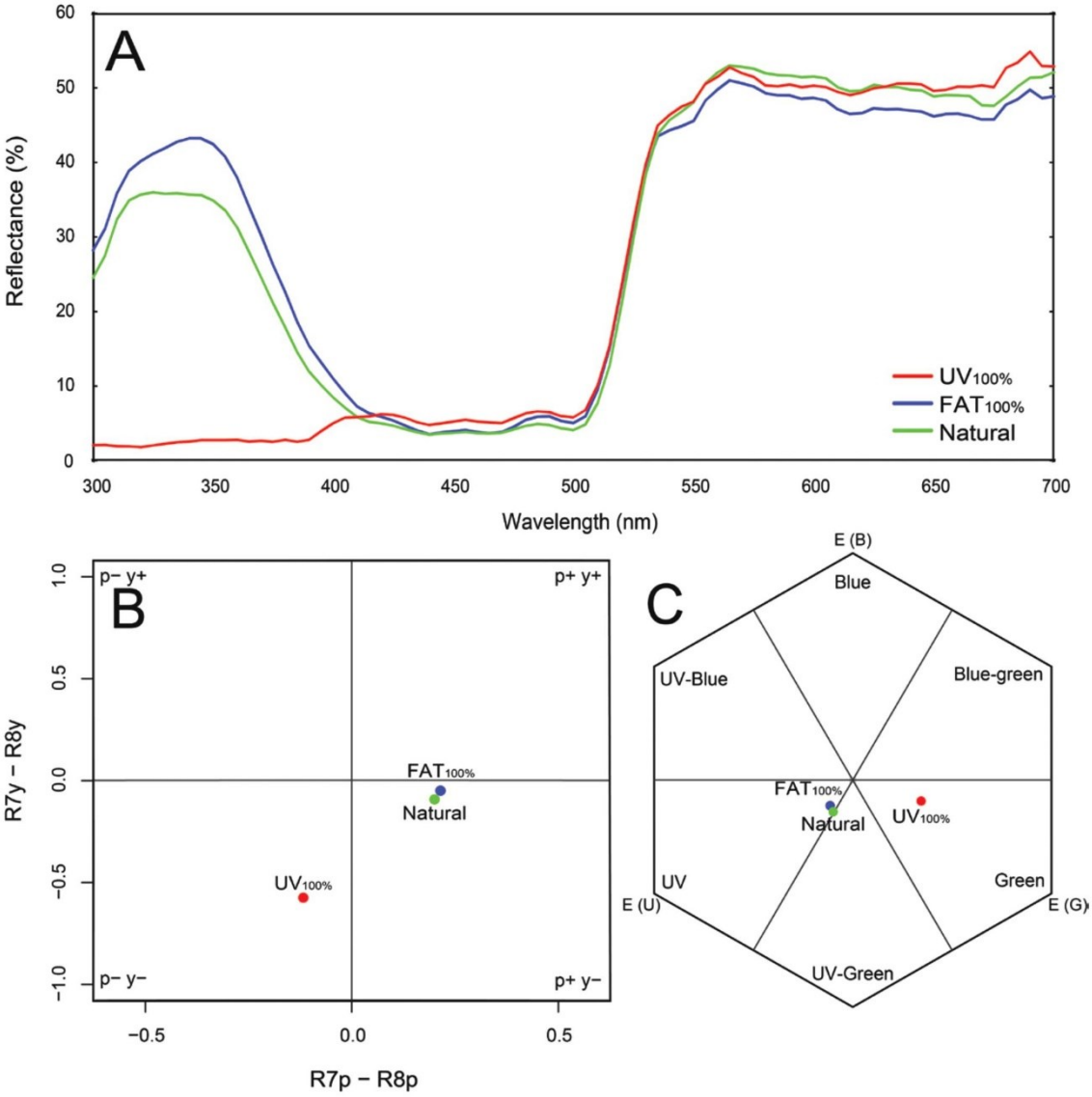

Figure 2. (A) reflectance of natural *H. camerooniana* flowers and those with experimental treatment and control (see Materials and Methods for more details). (B) Fly colour visual system displayed using the Troje model. (C) Bee colour visual system displayed using the colour hexagon model.

with the notable exception of *H. decumbens*, pollinated by flies in Venezuela (Raimúndez and Ramirez 1998). In addition to the previous studies of the genus based on visitation rates, we have confirmed that bees are the most efficient pollinators of *H. camerooniana* since: (i) bees were the most common visitors; (ii) in a large percentage of visits they are in contact with reproductive organs, which is expectable for these voracious pollen feeders; and (iii) their visits significantly increased numbers of germinated pollen tubes in stigmas. Contrary to Johnson and Andersson's (2002) observations on *H. hemerocallidea* in South Africa, there were only few visits of honeybees compared to the abundant visits by a single morphospecies of small solitary bees. Moreover, honeybees seemed to be mostly searching for nectar, although we did not observe any nectar in flowers of *H. camerooniana*, consistent with other *Hypoxis* species (Johnson and Andersson 2002; Rudall 2002; Ren *et al.* 2019).

UV and visitor frequency

The UV signal of flowers influenced the particular visitor frequency in different ways. Bees visited flowers more often when at least half of the petals reflected UV. However, although not significant, even the control flowers treated with non-absorbing cream differed in bee (but not fly) visitation frequencies from untreated flowers. The drop in visitation frequency between the treatments and their respective controls is consistent with the previous study of *H. hemerocallidea* (Johnson and Andersson

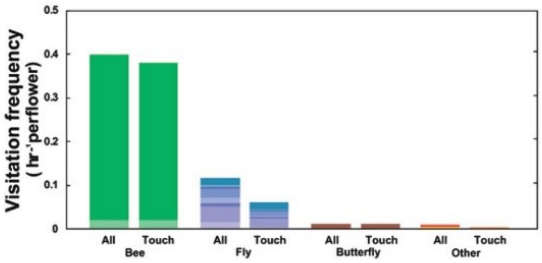

Figure 3. Frequency of all *H. camerooniana* flower visitors and visitors in contact with reproductive organs during daytime. Different colours indicate different morphospecies.

Table 1. Proportion of visits during which bees and flies touched reproductive organs of *H. camerooniana*. See Materials and Methods for the description of treatments.

	Bees		Flies	
Treatment	Stigma	Anthers	Stigma	Anthers
$UV_{100\,\%}$	85.7	92.9	0.0	57.1
$F_{100\,\%}$	76.7	95.3	15.4	23.1
$UV_{50\,\%}$	59.4	90.6	28.6	57.1
$F_{50\,\%}$	84.8	97.0	47.6	57.1
Natural	74.3	95.7	45.5	63.6

2002) in which fewer honeybees (*A. mellifera*) visited flowers with the floral UV reflectance obscured. While in alpine communities of New Zealand, Campbell *et al.* (2010) found experimentally manipulated flower colour, and not UV reflectance, to be more important for the visitation rates, other studies on UV pollinator visitation preferences showed similar results to ours (Peter and Johnson 2008; Rae and Vamosi 2013; Horth *et al.* 2014; Koski and Ashman 2014). Ultraviolet reflectance was revealed as highly important for bees pollinating *Eulophia zeyheriana* (Orchidaceae; Peter and Johnson 2008), whereas the general visitation rates of various visitors declined after manipulation of UV reflectance in two *Rudbeckia* species (Horth *et al.* 2014), *Mimulus guttatus* (Phrymaceae; Rae and Vamosi 2013), and *Argentina anserine* (Rosaceae; Koski and Ashman 2014). Therefore, UV reflectance plays an important role in pollinator attraction, but it can differ among flowering species, since other floral traits, such as scent, shape and colour, could be equally important.

Additionally, we have shown that having at least some UV reflectance is more important for selection of *H. camerooniana* flowers by bees than its UV pattern (*sensu* Koski and Ashman 2014), as flowers with fully covered petals by the UV-absorbing cream differed in bee visitation from those with all petals fully reflecting UV. Flies, however, although also showing a higher visitation frequency when the flower is fully or partially reflecting UV, did not significantly differentiate between $UV_{100\,\%}$ and the *Natural control*. These results are discordant with the previous study of yellow UV-reflecting flowers of *A. anserine* (Koski and Ashman 2014) which demonstrated that the presence of UV pattern increased visitation by both bees and syrphid flies relative to both fully UV reflective or absorptive flowers. Nevertheless, bees were repeatedly described to prefer flowers with some colour pattern above the unicoloured ones (e.g. Waser and Price 1985; Papiorek *et al.* 2016).

It must be stipulated that UV reflectance is just one channel of communication of plants with insects (Chittka *et al.* 1994; Johnson and Andersson 2002). Additionally, other traits or factors need to be considered when looking at the pollination system. For example, the community context (Peter and Johnson 2008; Campbell *et al.* 2010), positioning of flowers and inflorescences (Lunau 1992b; Johnson *et al.* 2003a, b; Rae and Vamosi 2013; van der Kooi 2016), other optical principles of flower colouration (e.g. van der Kooi *et al.* 2014, 2016) and learning ability of visitors (Giurfa *et al.* 1995; Hammer 1997; Dyer *et al.* 2015). The learning ability of visitors is difficult to include into such field experimental studies. Supplementary controlled studies with naïve bees would be greatly beneficial to further disentangle the factors important in shaping the studied pollination system.

UV and bee behaviour

The significant difference in the bee landing behaviour implies that the floral UV pattern can play an important role in orientation of bees on visited flowers. On the flowers completely covered with the UV-absorbing cream, bees mostly landed directly on the anthers and immediately started to collect pollen, whilst they landed mostly on petals of the flowers that at least partly reflected UV (i.e. all other treatments). This has proven that a disturbance of the UV pattern may change bees' behaviour. Likewise, other colour patterns, such as floral guides or bullseye patterns, are considered to increase the plants' reproductive success by helping pollinators to orientate to the flower centre (Waser and Price 1985; Dinkel and Lunau 2001; Leonard and Papaj 2011; Papiorek *et al.* 2016). However, bees actually make their first antennal contact preferably at the UV-absorbing floral area, irrespective of its spatial position within a flower (Papiorek *et al.* 2016). Therefore, one would expect bee visitors of *H. camerooniana* to prefer the centre of flowers with the UV-absorbing anthers, which is not the case. We thus hypothesize that in *H. camerooniana*, the UV-reflecting petals probably act as a landing platform, making flowers more visible for potential pollinators in its typical habitat of burnt montane grasslands, since the general UV reflection of similar grasslands vegetation is low (<5 %; Caldwell *et al.* 1983).

Methodological biases of UV manipulation

When Johnson and Andersson (2002) used the genus *Hypoxis* for the experimental manipulation of floral UV reflectance to study the response of insect pollinators, they did not include the experimentally untreated plants (*Natural control*). They thus did not control for the effect of experimental manipulation on natural insect behaviour. In our experiment, which based the methodology largely on the referred study, we demonstrated that such experimental setting is useful to investigate the effect of floral UV signalling on visitors. But at the same time, we discovered that experimental controls (i.e. flowers covered by the non-UV-absorbing cream compound; $FAT_{100\,\%}$, $FAT_{50\,\%}$) can differ from the untreated natural flowers. The experimental controls showed lower (but not statistically significant) visitation rates than the natural control for bees. Furthermore, the numbers of germinated pollen tubes on stigmas of natural control flowers of *H. camerooniana* were significantly higher compared to all treated flowers, apart from the control with fully covered non-UV-absorbing cream. These lower visitation rates and lower number of germinated pollen tubes could be explained by several factors, e.g. less evaporation of scents or changes in the glossiness of the flower. It also proved that we did not cause pollination during handling of experimental flowers.

Additionally, we showed that this effect can be visitor-specific. Flies, generally a more olfactory-oriented group than bees

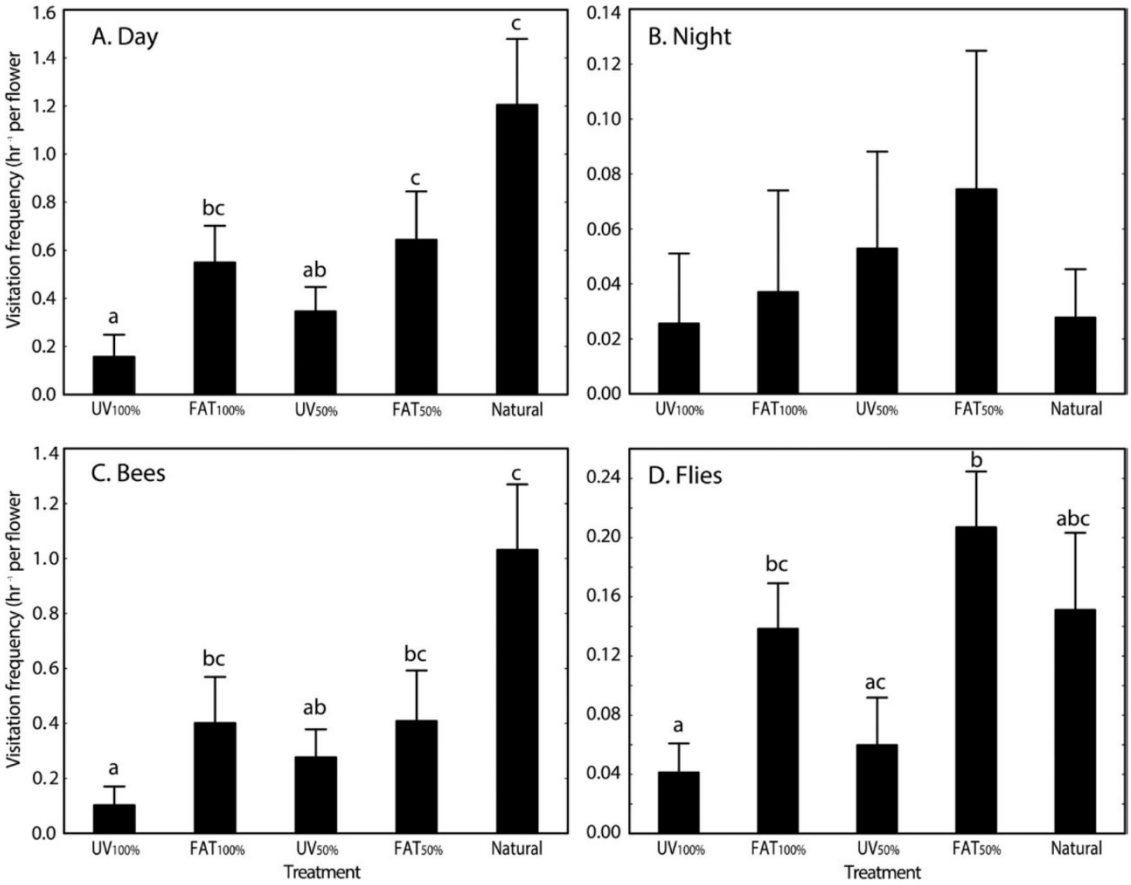

Figure 4. Effect of UV pattern manipulation on visitation frequencies of *H. camerooniana* for (A) day; (B) night; (C) bee; and (D) fly visitation during the day separately. Note: scaling of the Y-axis is not standardized due to the substantially lower number of visits between day and night, and between bees and flies. Means (bars) and SE (whiskers) are shown. The same letters above the columns indicate non-significant differences in the pairwise *post hoc* tests. See Materials and Methods for the description of treatments.

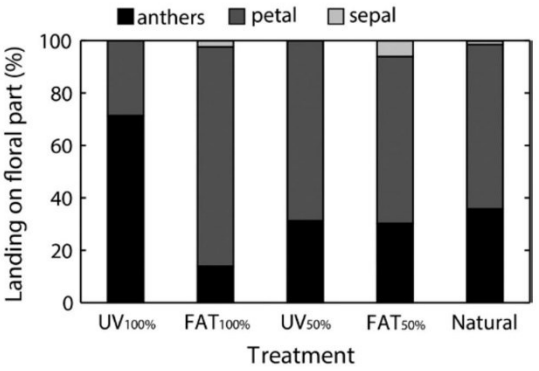

Figure 5. Effect of UV pattern manipulation on bee landing behaviour of *H. camerooniana* flowers. See Materials and Methods for the description of treatments.

(Roy and Raguso 1997), were not affected by the experimental controls at all. For this group, the UV manipulation treatments ($UV_{100\%}$, $UV_{50\%}$) did not significantly differ from the *Natural* control.

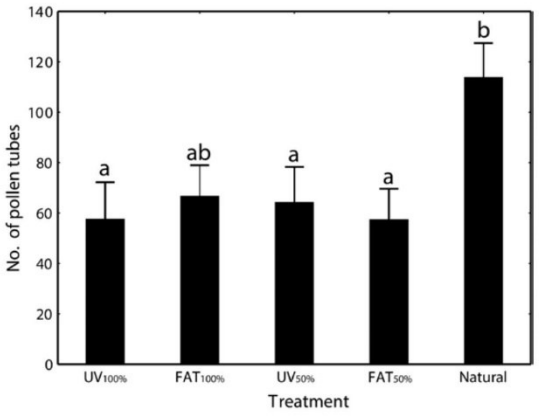

Figure 6. Effect of UV pattern manipulation on the number of germinated pollen tubes in stigmas of *H. camerooniana*. Means (bars) and SE (whiskers) are shown. The same letters above the columns indicate non-significant differences in the pairwise *post hoc* tests.

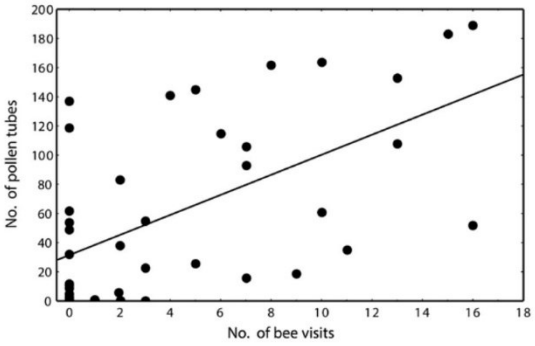

Figure 7. Effect of number of bee visits on number of germinated pollen tubes in stigmas of *H. camerooniana*.

Although, they were visited by significantly less flies than the controls ($FAT_{100\ \%}$, $FAT_{50\ \%}$). We thus speculate that the effect of UV manipulation is at least partly compensated by the potential attraction of Muscidae and Sarcophagidae flies (which created most of the diversity of recorded fly visitors) to the duck preen gland fat. Bees, as generally more visually oriented insects, expressed higher, but still non-significant, differences among natural and experimental controls. Furthermore, the thickness of the cream layer or amount of cream in the treatments has not been considered in our study but might play a role in the attraction of flies.

In summary, although the duck preen gland fat is a commonly used vector for the UV-manipulating agents (e.g. Johnson and Andersson 2002; Peter and Johnson 2008; Welsford and Johnson 2012; Rae and Vamosi 2013), it affects natural insect behaviour. Consequently, we strongly encourage 'calibration' of results by controlling for the chemical vector's (duck preen gland fat in our case) effects in similar experimental studies.

Conclusion

The primary pollinators of *H. camerooniana* in the Afromontane grasslands of Mount Cameroon were bees. When UV reflectance was completely removed visitation rates of bees decreased, whereas the decrease of frequency on half-treated flowers was not significant (although it decreased as well when considering all daytime visitors). The complete UV reflectance removal changed the landing behaviour of bees as well, confirming that altering the natural UV patterns affects both visitation rates and behaviour.

Furthermore, based on our results we also encourage the inclusion of a natural control in the experimental designs of similar manipulative studies to control for the substances used in floral manipulation.

Supporting Information

The following additional information is available in the online version of this article—

Figure S1. Frequency of nocturnal visits of *Hypoxis camerooniana* flowers.

Figure S2. Diurnal changes in bee visitation frequencies on flowers of *Hypoxis camerooniana* after manipulation of their ultraviolet (UV) reflectance. Means (bars) and SE (whiskers) are shown.

Figure S3. Diurnal changes in fly visitation frequencies on flowers of *Hypoxis camerooniana* after manipulation of their ultraviolet (UV) reflectance. Means (bars) and SE (whiskers) are shown.

Figure S4. Bee (A) and fly (B) behaviour on flowers of *Hypoxis camerooniana* after manipulation of their ultraviolet (UV) reflectance. There was no significant effect of treatment on both bee ($F_{ps} = 0.49$; df = 4; $P_{perm} = 0.770$) and fly ($F_{ps} = 0.44$; df = 4; $P_{perm} = 0.903$) behaviour after landing.

Data

An excel file with the data used for analyses and graphs is available in the online version of this article.

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Contributions by the authors

All authors helped design the experiment, Y.K., R.D.K., M.B., J.E.J.M. and S.J. were involved in data collection; R.D.K., S.J., R.T. and Y.K. were in charge of data analyses and writing first drafts of the manuscripts. All authors contributed to writing and editing of the manuscript.

Conflict of Interest

None declared.

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Supplementary material

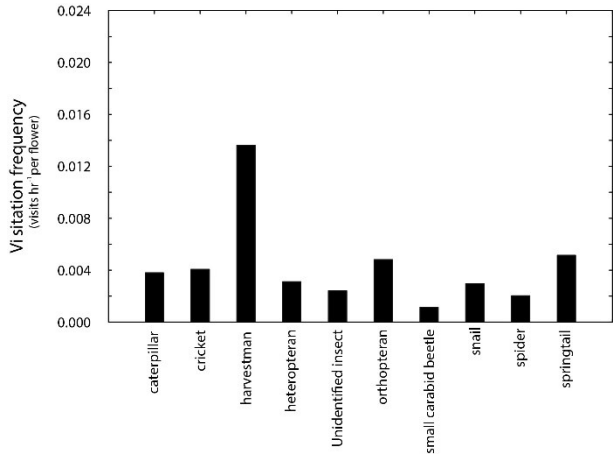

Fig. S1: Frequency of nocturnal visits of *Hypoxis camerooniana* flowers.

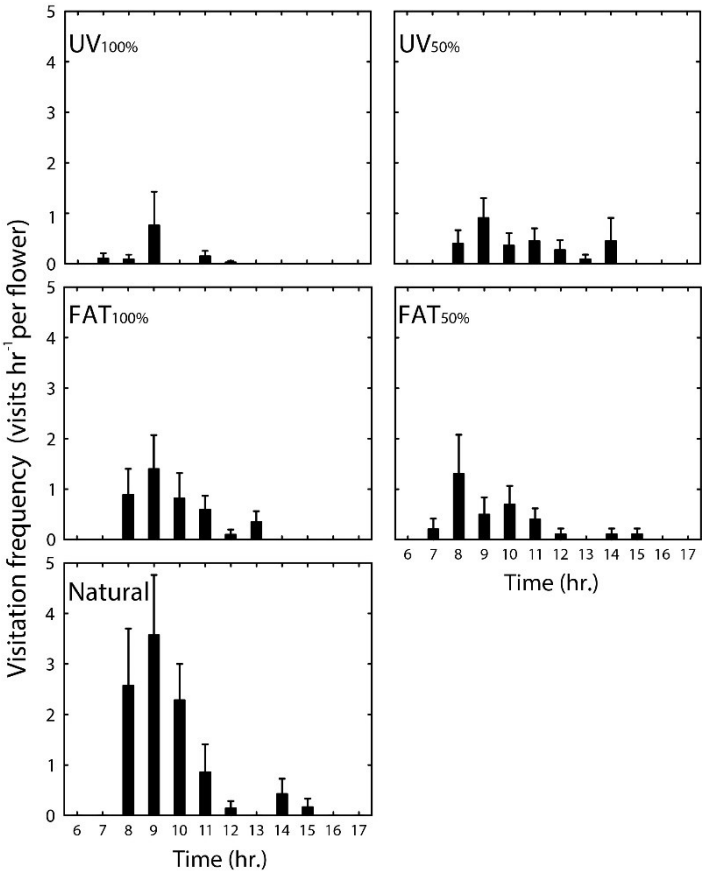

Fig. S2: Diurnal changes in bee visitation frequencies on flowers of *Hypoxis camerooniana* after manipulation of their UV reflectance. Means (bars) and SE (whiskers) are shown.

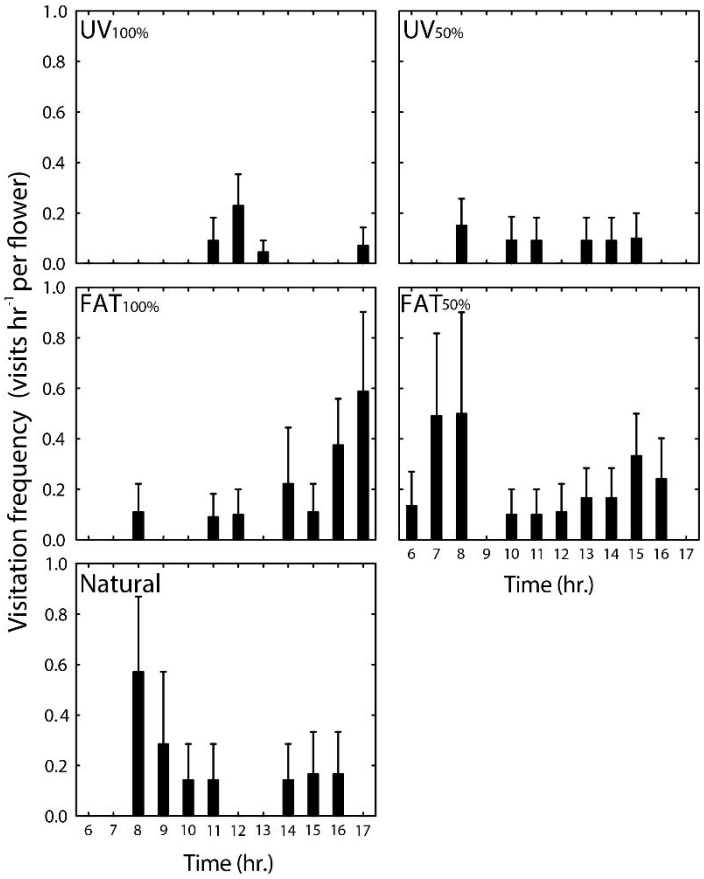

Fig. S3: Diurnal changes in fly visitation frequencies on flowers of *Hypoxis camerooniana* after manipulation of their UV reflectance. Means (bars) and SE (whiskers) are shown.

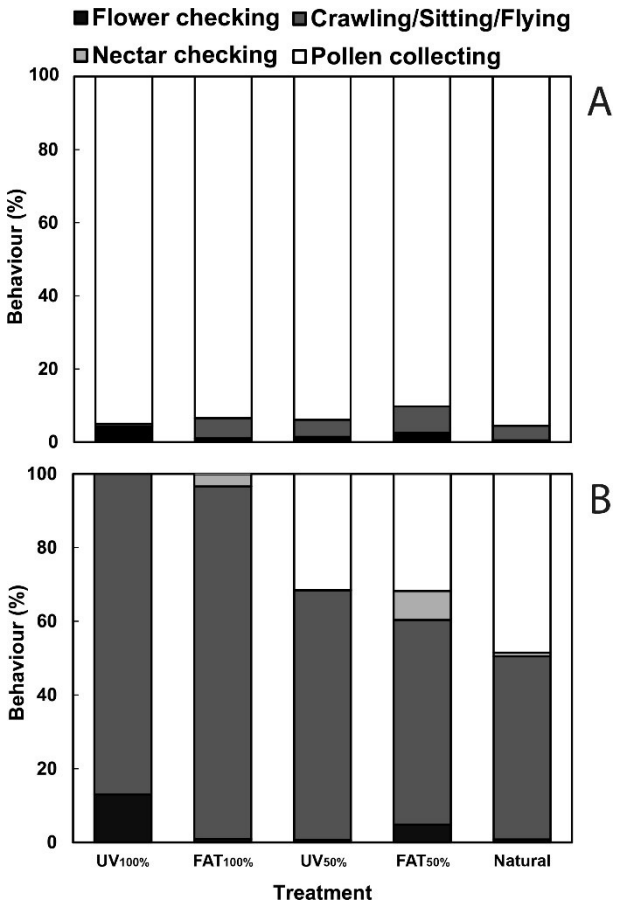

Fig. S4: Bee (A) and fly (B) behaviour on flowers of *Hypoxis camerooniana* after manipulation of their UV reflectance. There was no significant effect of treatment on both bee (F_{ps}=0.49; df=4; P_{perm}=0.770) and fly (F_{ps}=0.44; df=4; P_{perm}=0.903) behaviour after landing.

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